

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, 1/70, C07H 21/02, 21/04	A1	(11) International Publication Number: WO 96/30545 (43) International Publication Date: 3 October 1996 (03.10.96)
(21) International Application Number: PCT/US96/02045 (22) International Filing Date: 14 February 1996 (14.02.96) (30) Priority Data: 08/410,658 24 March 1995 (24.03.95) US (71) Applicant: APPLIED GENETICS, INC. [US/US]; Suite A, 11494 Sorrento Valley Road, San Diego, CA 92121 (US). (72) Inventors: FAHY, Eoin, D.; 7171 Canyon Hill Way, San Diego, CA 92126 (US). GHOSH, Soumitra; 13285 Deron Avenue, San Diego, CA 92129 (US). (74) Agents: MacWRIGHT, Robert, S. et al.; Kenyon & Kenyon, One Broadway, New York, NY 10004 (US).		(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MUTATION DETECTION BY DIFFERENTIAL PRIMER EXTENSION OF MUTANT AND WILDTYPE TARGET SEQUENCES		
(57) Abstract <p>The present invention provides methods for simultaneously interrogating two or more related polynucleotides. One embodiment of the present invention concerns a method for simultaneously analyzing a genetic mutation and a corresponding wild-type sequence within a sample. This method comprises: a) hybridizing a primer to a nucleic acid suspected of containing a genetic mutation, wherein the primer is hybridized 3' to the suspected mutation; b) extending the primer in the presence of a mixture of at least one deoxynucleoside triphosphate and at least one chain terminating dideoxynucleoside triphosphate selected such that the wild-type extension product and the mutant-DNA derived extension product have a total number of nucleotides that differ from one another and from the primer; c) separating the primer, the mutant DNA-derived extension product and the wild-type extension product on the basis of their size; and d) identifying the mutant DNA-derived extension product and the wild-type extension product.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

MUTATION DETECTION BY DIFFERENTIAL PRIMER EXTENSION OF MUTANT AND WILDTYPE TARGET SEQUENCES

FIELD OF THE INVENTION

The present invention relates generally to the simultaneous interrogation of related polynucleotides and, more particularly, to diagnostic methods for
5 detecting genetic mutations within genes implicated to play a role in the progression of particular diseases. In addition, the invention provides for a method of accurately quantifying the prevalence of a mutation in a background of a normal gene sequence.

10

BACKGROUND OF THE INVENTION

Inherited and acquired genetic disorders account for a large percentage of today's health care costs. Early diagnosis of such diseases is not only important
15 for successful treatment but also contributes to lower overall costs to the public. Lower costs result, because many diseases can be averted or even treated before chronic symptoms occur, which require expensive procedures and/or hospitalization.

20 Although significant advances have been made in the medical diagnostics field, many of these procedures are designed to detect significant differences in gene or protein structure. Where applicable, other procedures rely on the qualitative determination of the presence or
25 absence of a particular gene or gene product. Because of the large differences between the disease-associated gene and the normal gene, such diagnostic methods are

easily adapted to the diagnostic laboratory and to the skill level of the technician. However, the accurate and reproducible diagnosis of subtle genetic alterations, such as point mutations and the like within a disease-associated gene, is a significant challenge. There also exists a critical need for developing efficient genetic techniques for detecting mutations associated with drug resistance arising from anti-viral and anti-bacterial treatments, deleterious mutations in tumor suppressor genes, mutations in breast and ovarian cancer susceptibility genes, and for diagnosis of diseases of mitochondrial origin.

Mitochondrial genetics has become the subject of intense research because of its association with aging and late-onset degenerative diseases, such as Alzheimer's disease (AD). See Wallace, Proc. Natl. Acad. Sci. USA, 91: 8739-8746 (1994); Science, 246: 628-632, (1992). Mitochondrial genetic disease is characterized by 1) maternal inheritance, 2) heteroplasmy, wherein only a proportion of the mitochondrial DNA (mtDNA) is present in the mutant form, and 3) presentation of bioenergetic deficiencies which worsen with age and affect selective tissues, depending on their mitochondrial energy requirements. Wild-type and mutant mtDNA segregate randomly during mitosis and meiosis. Consequently, mitochondrial genetic disease can appear sporadically without discernable familial links, and the level of heteroplasmy can result in variable phenotypes and tissue expression. A nucleic acid-based analysis of mtDNA-associated mutations therefore faces the challenge of detecting and quantifying the degree of heteroplasmy of the mutations which may be present in very low proportions.

Several methods and strategies have been devised to analyze the presence of mutations within disease-associated genes and in genes responsible for the development of drug resistance.

Direct sequencing methods have been traditionally used for screening of mutations. However, such methods are costly, time consuming and require the analysis of multiple clones of the targeted gene for unambiguous
5 detection of low frequency mutations.

A variety of detection methods have been developed which exploit sequence variation in DNA using enzymatic and chemical cleavage techniques. Restriction fragment length polymorphism (RFLP) is a useful approach for
10 genetic analysis [see Botstein et al. in Am. J. Hum. Gen., 32: 314-331 (1980)], but its application is limited to detecting mutations that either create or disrupt recognition sequences of restriction enzymes. Both Rnase A cleavage and the chemical cleavage of
15 mismatch methods rely on enzymatic or chemical strand scission at mismatched base pairs to reveal the presence of mutations. These methods are limited by their high backgrounds, their inability to detect all base substitutions due to the nature of the unpaired bases,
20 the influence of local sequence environment on cleavage susceptibilities, the lack of information concerning the precise base change, and the difficulty in resolving similarly-sized cleavage products.

Electrophoretic methods used to detect mutations
25 include single strand conformational polymorphism, denaturing gradient gel electrophoresis (DGGE), and heteroduplex electrophoresis. Sequence variations are detected from differential electrophoretic gel mobilities resulting from subtle changes in tertiary
30 structure of wild-type and mutant molecules. The efficacy of these techniques is sequence dependent, since they rely on the influence of the mutation in altering the melting profile or the conformation of the molecule. Further, these methods are not informative
35 about the location and the nature of the nucleotide substitution.

Analysis of point mutations in DNA have been described using variants of the polymerase chain reaction (PCR). Gibbs et al. Nucl. Acids. Res., 17: 2437-2448, (1989); Newton et al. Nucl. Acids. Res., 17: 2503-2516, (1989). Mismatches are detected by competitive oligonucleotide priming under hybridization conditions where binding of the perfectly matched primer is favored, thereby providing a method of discrimination between normal and mutant sequences. These strategies require considerable optimization to ensure that only the perfectly annealed oligonucleotide functions as a primer for the PCR reaction.

In enzyme-mediated ligation methods, mutations are detected when oligonucleotide sequences annealed immediately adjacent to each other on a target DNA or RNA molecule are covalently attached only if oligonucleotides are correctly base-paired. [Grossman et al., Nucleic Acids Research, 22: 4527-4534, (1994)] The usefulness of the method is sometimes compromised by high backgrounds which can arise from tolerance of certain nucleotide mismatches or from non-template directed ligation reactions [Barringer, et al. Gene, 89: 117-122, (1990)].

Single base mutations in target nucleic acids have been detected by differential hybridization techniques using allele-specific oligonucleotide (ASO) probes. Saiki et al. Proc. Natl. Acad. Sci. USA, 86:6230-6234, (1989). Mutations are identified on the basis of the higher thermal stability of the perfectly matched probes as compared to the mismatched probes. This approach has several disadvantages for multiple mutation analysis: (1) the technique requires optimization of the hybridization parameters for each probe, and (2) the nature of the mismatch and the local sequence impose limitations on the degree of discrimination of the probes.

Single nucleotide primer-guided extension assays have been used for detecting aspartylglucosaminuria, hemophilia B and cystic fibrosis mutations genotyping of apolipoprotein E, and for quantifying point mutations associated with Leber Hereditary Optic Neuropathy (LHON). Examples of the above applications are found in the following: Kuppuswamy et al. Proc. Natl. Acad. Sci. USA, 88: 1143-1147, (1991); Syvanen et al. Genomics, 8: 684-692, (1990); Juvonen et al. Human Genetics, 93: 16-20, (1994); Ikonen et al. PCR Meth. Applications, 1: 234-240, (1992); Ikonen et al. Proc. Natl. Acad. Sci. USA, 88: 11222-11226, (1991); Nikiforov et al., Nucleic Acids Research, 22: 4167-4175 (1994). The high fidelity of DNA polymerases ensures specific incorporation of the correct base labeled with a reporter molecule and enables quantification of heteroplasmy not readily achieved by other analytical methods. These methods typically use radiolabeled nucleotides for detection and are limited in scope due to low throughput and difficulty in automation. Since these methods interrogate each mutation site for the presence of wild-type and mutant nucleotide in separate reactions, monitoring 20 codon sites would minimally require 40 different reactions. Only limited multiplexing of the assays is possible using different reporter labels for the four bases.

The single nucleotide primer extension reaction approach has been modified for detection of multiple mutations. A. Krook et al., Human Molecular genetics, 1: 391-395 (1992). Multiplexing is achieved by using primers of different lengths and by monitoring the wild-type and mutant nucleotide at each mutation site in two separate single nucleotide incorporation reactions. The reaction mixtures are resolved by gel electrophoresis and the identity of the nucleotide in the mutation site is determined by the presence of a correct size band in the wildtype or mutant nucleotide lanes. Multiplexing

for population screening is achieved by pooling PCR amplified products from individual patients, with each pool consisting of 5-10 subjects. A positive pool from the first round of screening is then deconvoluted by
5 analyzing the amplified PCR product of each individual in the pool.

Use of the primer extension technique for nucleic acid detection and mutational analysis is described in PCT WO 90/09455. The outlined methods include (1) a
10 solid support format, e.g., by using a detection label and biotin-avidin affinity purification step, and (2) a gel electrophoresis approach for separation of products. Several of the embodiments describe multiplexed detection of different target sequences or presence of
15 specific nucleotides in different target sequences using differentiable primers. This reference, however, does not address the simultaneous interrogation of related polynucleotides, such as mutant and wild-type sequences.

Thus, there exists a need for a diagnostic method
20 for simultaneously interrogating related polynucleotides such as wild-type and mutant genes, particularly rare genes with multiple mutations. The method should be amenable to automation with the capabilities of non-isotopic detection, high throughput, multiple codon
25 analysis, convenient readout, and finally quantification of the level of heteroplasmy.

These and other needs are met by the present invention.

30 SUMMARY OF THE INVENTION

The method of the present invention is based on polymerase-directed extension of an oligonucleotide primer using selected mixtures of up to three nucleoside triphosphates and one or more chain terminating, base
35 pairing entities, such as dideoxynucleoside triphosphates.

An embodiment of the present invention concerns a method for simultaneously analyzing a genetic mutation and a corresponding wild-type sequence within a sample. This method comprises: a) hybridizing a primer to a
5 nucleic acid suspected of containing a genetic mutation, wherein the primer is hybridized 3' to the suspected mutation; b) extending the primer in the presence of a mixture of at least one deoxynucleoside triphosphate and at least one chain terminating dideoxynucleoside
10 triphosphate selected such that the wild-type extension product and the mutant-DNA derived extension product have a total number of nucleotides that differ from one another and from the primer; c) separating the primer, the mutant DNA-derived extension product and the wild-
15 type extension product on the basis of their size; and d) identifying the mutant DNA-derived extension product and the wild-type extension product.

Another embodiment of the invention concerns a method for simultaneously determining the presence of
20 related polynucleotide sequences in a nucleic acid sample. This embodiment comprises the steps of: a) providing a nucleic acid sample of a type known to contain at least two related polynucleotide sequences, each having an identical region and a divergent region,
25 the identical regions having identical nucleotide sequences terminating at their 5' ends at the divergent regions, wherein identity between the at least two polynucleotide sequences ceases; b) providing a primer that is complementary to the identical regions; c)
30 hybridizing the primer to the identical regions; d) extending the primer into the divergent regions in the presence of a polymerase and a nucleotide mixture containing at most three dNTPs, such that an extension product of unique length is formed for each of the
35 related polynucleotide sequences; and e) separating the primer and the extension products based on their respective lengths.

Many variations upon these embodiments are possible. For example, the nucleotide mixture can contain one to three dNTP's and one to three chain terminating, base-pairing entities. Alternatively, the
5 nucleotide mixture can contain two or three dNTP's and no chain terminating, base-pairing entities.

In addition, the extending step can be repeated one or more times to increase the amount of extended product. Alternatively, the nucleic acid suspected of
10 containing the mutation can be the product of PCR or RT-PCR.

The method can be multiplexed by using at least two primers that can be differentiated, for example, by means of differing length or fluorescent labels.
15 Depending on the desired extension products, the primers can be extended in a single reaction using a compatible nucleotide mixture or extended in separate reactions using different nucleotide mixtures which are combined before separating the extension products.

20 Other embodiments of the invention concern kits containing one or more primers, a polymerase, and one or more of the nucleotide mixtures discussed above.

It is an object of the present invention to provide a diagnostic method for simultaneously detecting related
25 polynucleotides such as wild-type and mutant genes.

It is yet another object of the present invention to provide a method that can be easily multiplexed for the analysis of multiple loci within a given nucleic acid sample.

30 It is a further object of the present invention to diagnose inherited and acquired genetic disorders in their early stages, improving the chances of successful treatment and contributing to lower overall costs to the public.

35 It is another object of the present invention to accurately diagnose subtle genetic alterations, such as

point mutations and the like, within a disease-associated gene.

It is a further object of the present invention to quantify the degree of heteroplasmy of a given mutation, even if that mutation is present in very low proportions.

It is another object of the present invention to probe multiple mutations using the same reaction mixture.

10 It is a further object of the present invention to provide a method that is amenable to automation with the capabilities of non-isotopic detection, high throughput, multiple codon analysis, and convenient readout.

One advantage of the present invention is that it is amenable to a wide range of potential applications including detection of mtDNA mutations associated with AD and LHON, rare somatic mutations, cystic fibrosis mutations, multiple mutations in HIV-1 pol and protease genes associated with drug resistance, p53 gene mutations and predisposing mutations in the breast and ovarian cancer susceptibility gene BRCA1 and BRCA2.

Another advantage of the present invention is that all extension reactions of a specific primer are conducted in a single reaction mixture. Accordingly, relative amounts of the various extension products will correspond to their complements found within the nucleic acid sample. In other words, no tube-to-tube variations, such as variations in concentration or priming, will distort analysis of the relative amounts of the extension products.

Yet another advantage of the present invention is that it is amenable to automation.

The appended claims are hereby incorporated by reference as a further enumeration of preferred embodiments. Other objects and advantages of the invention and alternative embodiments will readily become apparent to those skilled in the art,

particularly after reading the detailed description, and examples set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a schematic representation for the simultaneous interrogation of a wild-type sequence and two mutant sequences using the primer extension method. Single base substitutions in codon 95 (leucine, CTT) of the mtDNA-encoded cytochrome c oxidase subunit 2 (COX2) gene result in codons for proline and phenylalanine (CCT and TTT, respectively). Polymerase extension of a primer having an A base at its 3' terminus with ddGTP and dATP in the reaction mix results in the synthesis of three extension products. The extension product derived from the wild-type template is two nucleotides longer than the primer, resulting from incorporation of dA followed by ddG chain termination. The mutant template coding for proline directs the synthesis of an extension product which is a single nucleotide longer than the primer, and which results from chain termination with ddG. The product from the template coding for phenylalanine is formed by addition of two dA residues followed by chain termination with ddG. The three products, differing in size from each other and the primer, are easily separated by gel electrophoretic techniques. The mobilities of the extension products relative to the primer are diagnostic of the sequence being analyzed.

Figure 2 shows quantification of the level of a mutation within a particular sample. Plasmid DNA (10^{-17} mol) containing mutant and wild-type plasmid mixtures (COX2 genes, codons 20 and 90) were amplified by PCR and aliquots of the purified products were used in ULTma™ DNA polymerase-catalyzed primer extension reactions. The nucleotide combinations were designed such that the extension products derived from the wild-type templates are one base longer than those derived from the mutant

templates. For each codon the lanes are represented as follows: Lane 1: wild-type; lane 2: mutant; lane 3: 10% mutant; lane 4: 5% mutant; lane 5: 1% mutant.

Figure 3 is a simulation of a polyacrylamide gel pattern showing the simultaneous analysis by primer extension of multiple mutations in the COX1 and COX2 genes. Two or more primers differing in length by at least 5 bases are used in the same extension reaction to interrogate 2 or more codons on the same target sequence. Furthermore, multiplexed primer extension reactions may be loaded on the same gel at different time intervals to increase throughput. In this example, 3 multiplexed reactions for both the COX1 and COX2 targets are loaded on the same lanes at 3 different time points, allowing for the simultaneous analysis of a total of 14 codons. The tick-mark interval on the X-axis represents 1 nucleotide.

Figures 4A and 4B are schematic representations of sequence discrimination by primer extension in the presence of 3' deoxynucleotides and chain terminating dideoxynucleotides. Figure 4A shows the nucleotide combinations for analysis of both the wild-type and mutant sequence at COX 2, codon 20. The primer used is 24 nucleotides long and terminates with a dA residue at its 3'-end. Figure 4B shows the nucleotide combination for analysis of both the wild-type and mutant sequence at COX 2, codon 95. The primer used is 20 bases long and has a dA residue at its 3'-terminus.

Figures 5A-5C show the analysis of multiple codons for the presence of a suspected mutation in a single primer extension reaction using a compatible mix of deoxynucleotides and chain terminating dideoxynucleotides. Figure 5A uses Vent[®] DNA polymerase with dATP and ddGTP for analyzing COX2, codons 20 and 95. Figure 5B shows the use of Taq DNA polymerase with dATP and ddGTP in the primer extensions for analysis of COX2 codons 20 and 95. Figure 5C shows the use of

ULTma™ DNA polymerase with dATP and ddGTP in the primer extensions for analysis of COX2, codons 20 and 95.

Figure 6 is a schematic representation of sequence discrimination by extension in the presence of deoxynucleotides and chain terminating dideoxynucleotides. The mutation analyzed is the nucleotide 3460 mutation in the ND1 mitochondrial gene which is associated with Leber Hereditary Optic Neuropathy (LHON). Two combinations of dNTPs and ddNTPs are used to independently verify presence of the mutations. The first combination (dCTP, ddGTP, ddTTP) provides a wild-type-derived extension product which is longer by one base than the mutant-derived extension product. The converse case results from using the second combination (dTTP, ddGTP, ddCTP).

Figure 7 is a standard curve for quantification of a mutation at codon 71 of the COX2 gene.

Figure 8 illustrates mutation analysis by gel electrophoresis of codon 415 of the COX1 gene for 60 patient samples using multiple sample loadings.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to a rapid, sensitive and efficient method for simultaneously interrogating and quantifying related polynucleotides in a nucleic acid sample. The method is particularly applicable for detecting rare genetic mutations and quantifying the level of heteroplasmy within a sample, using a highly sensitive non-radioisotopic protocol.

The primer extension method described herein generates extension products of different lengths from related polynucleotide templates, such as wild-type and one or more mutant templates, using a primer, a polymerase enzyme, appropriate combinations of dNTPs and, preferably, ddNTPs. The extension products are easily resolved based on their molecular weights and, in the event the extension products are labeled with a

detection moiety, quantified by measurement of the label. Differences in length of only a single nucleotide are detected in the method of the present invention. For example, as can be seen below in Example 5 III and Figure 5A, a primer of 24 nucleotides in length, a wild-type extension product of 26 nucleotides in length and a mutant extension product of 25 nucleotides in length can be easily resolved. This primer and these extensions products are said to differ from each other in total nucleotides (i.e., length) by at least one nucleotide. In this case, each of the primer and the extension products is said to have a "different" or a "unique" number of nucleotides.

In order to facilitate a full and complete understanding of the present invention, it is important to note that all terms used herein are intended to have the same meaning as generally ascribed to those terms by those skilled in the art of molecular genetics, unless defined to the contrary. The references cited herein are incorporated by reference in their entireties.

In using the terms "nucleic acid", RNA, DNA, etc., we do not mean to limit the chemical structures that can be used in particular steps. For example, it is well known to those skilled in the art that RNA can generally be substituted for DNA as a template, and as such, the use of the term "DNA" should be read by those skilled in the art to include this substitution. In addition, it is known that a variety of nucleic acid analogues and derivatives can be made and will hybridize to one another and to DNA and RNA, and the use of such analogues and derivatives is also within the scope of the present invention.

As used herein, the term "related polynucleotides" refers to two or more polynucleotides, each having identical regions of identical nucleotide sequence. The "identical regions" are those regions where there is absolute identity between the related polynucleotides.

The first nucleotides adjacent to the 5' ends of the identical regions (wherein identity ceases) are the first nucleotides in the "divergent regions." Divergent regions can arise from the deletion, addition or substitution of one or more nucleotides. Divergent regions consist of one or more nucleotides. For convenience, the nucleotide at the 3' end of the divergent regions wherein identity between the polynucleotides ceases is referred to herein as "the point of deviation" of the related polynucleotides. For the effective operation of the present invention, the identical regions of the two or more polynucleotides should contain a sufficient number of bases to ensure specific hybridization of a complementary primer.

As used herein, the term "genetic mutation" refers to a change (or changes) in a nucleotide sequence of a gene or related region that is different from the normal or wild-type sequence. Mutations include, for example, substitutions, additions and deletions within the wild-type sequence. Such substitutions, additions or deletions can be single nucleotide changes such as occurs in a point mutation or they can be two or more nucleotides which may result in substantial changes to the gene sequence or structure. Mutations can occur within the coding region of the gene as well as within the non-coding and regulatory regions. The term is intended to include silent and conservative mutations within the gene's coding regions as well as changes which alter the amino acid sequence of the protein product.

As used herein, the term "primer extension" refers to polymerase-mediated 3'-extension of a priming nucleic acid sequence which is annealed to a nucleic acid template. The template can be, for example, DNA, RNA or their analogs. Such polymerization of the primer results in the synthesis of a complementary copy of the template sequence. Primer extensions can be performed

using, for example, DNA- or RNA-directed polymerases, depending on the template to be copied. The term "high fidelity" when used in reference to a polymerase is intended to mean those polymerases which exhibit 3'-5' exonuclease activity and concomitant proof-reading function.

As used herein, the term "deoxynucleotide" or "deoxynucleoside triphosphate" refers to a 2'-deoxynucleoside triphosphate containing either of the bases adenine, cytosine, guanine or thymidine or functional equivalents thereof. The abbreviations used for the above nucleotides are dATP, dCTP, dGTP and dTTP, respectively. Collectively, they are abbreviated as dNTP's. Once incorporated into a given sequence, they are simply abbreviated as dN's. These abbreviations are standard to those skilled in the art.

The term "chain terminating, base pairing entity" refers to a nucleoside triphosphate containing either of the bases adenine, cytosine, guanine or thymidine or functional equivalents thereof in which hydroxyl groups are absent at the 3' position of the sugar moiety. The term "chain terminating dideoxynucleotide" or "dideoxynucleoside triphosphate" refers to a nucleoside triphosphate containing either of the bases adenine, cytosine, guanine or thymidine or functional equivalents thereof, which are missing hydroxyl groups at the 2' and 3' positions of the ribose moiety. For DNA, the abbreviations used for the dideoxynucleotides are ddATP, ddCTP, ddGTP and ddTTP, respectively. Collectively, they are abbreviated as ddNTP's. Once incorporated into a given sequence, they are simply abbreviated as ddN's. The abbreviations are standard to those skilled in the art.

As used herein, the terms "detection moiety" and "reporter molecule" refer to a specific moiety or chemical structure which facilitates detection of the primer extension products. Such moieties can be, for

example, fluorescent, luminescent or radioactive labels, enzymes, haptens and other chemical tags such as biotin which allow for easy detection of extension products. Fluorescent labels such as the dansyl group, fluorescein and substituted fluorescein derivatives, acridine derivatives, coumarin derivatives, phthalocyanines, tetramethylrhodamine, Texas Red®, 9-(carboxyethyl)-3-hydroxy-6-oxo-6H-xanthenes, DABCYL® and BODIPY® (Molecular Probes, Eugene, OR), for example, are particularly advantageous for the methods described herein. Such labels allow for quantitative detection of the extension products and can be routinely used with automated instrumentation for simultaneous high throughput analysis of multiple samples. One skilled in the art will know or can readily determine what type of detection moiety to use for a particular application.

The present invention provides a method for simultaneously determining the presence of at least two related polynucleotides (e.g., a wild-type and a mutant gene) having a known nucleotide sequence. In general, the method includes a) hybridizing a primer to each of the related polynucleotides at a position that is proximal and 3' to the point of deviation between the related polynucleotides, b) extending the primer in the presence of a mixture that contains either i) two or three dNTPs and no chain-terminating, base-pairing entities or ii) one to three dNTPs and one to three chain-terminating, base-pairing entities, wherein the specific mixture is designed based on knowledge of the deviations between the related polynucleotides to ensure that each related polynucleotide produces an extension product that differs in length from the extension products of the other related polynucleotides and from the primer (for specific embodiments, see, e.g., Table I), and c) separating the extension products from one another and from the primer based on the relative size of each (e.g., on the basis of molecular weight).

The method of the present invention is typically used in connection with a genetic mutation within a gene. However, the method of the invention is useful in any instance where related polynucleotides are to be analyzed. Thus, to the extent that the following discussion is directed to genetic mutations, the present invention should not be construed as being limited to such.

Samples to be analyzed by the method of the invention are obtained by any method known to those skilled in the art. The sources for such samples includes blood cells, other cell types within an organism, or essentially any other source of genetic material. The isolation of genetic material is routine and can be performed by one skilled in the art using a variety of methods well known in the art. Such methods include cell lysis by freeze-thaw, proteinase K digestion, followed by phenol/chloroform extraction of DNA. Known non-organic techniques include cell lysis and proteinase K digestion, followed by purification of the DNA by QIAamp™ extraction columns (Qiagen, Chatworth, CA). Alternatively, isolation can be circumvented by procedures which allow the reproduction and/or amplification of the nucleic acid suspected of containing the genetic mutation. The polymerase chain reaction (PCR), or comparable methodology, is particularly applicable for this alternative approach, because it allows the target sequence to be directly amplified from as little as a single cell of starting material. The reverse transcriptase-polymerase chain reaction (RT-PCR) would be applicable if it is desirable to amplify the target sequence from RNA. Amplification procedures which allow for the direct isolation of PCR products can additionally be employed to increase the overall efficiency of the procedure. Direct isolation can be accomplished, for example, by employing PCR primers which are modified to contain a tag such as

biotin which can be used to isolate one or more strands of the PCR product away from other components of the reaction mixture.

Once the nucleic acid of interest is isolated
5 and/or amplified, it is hybridized to a primer to yield a primer-template that is used as a polymerase substrate. The primer is preferably designed to satisfy at least two criteria. The first criterion is that the primer be capable of specifically hybridizing to the
10 target nucleic acid sequence. Specific hybridization of the primer and target sequence is achieved when undesired cross-hybridization with other sequences is not observed. The second criterion is that the primer is hybridized proximal and 3' to the point of deviation
15 between the related nucleotides. By "proximal" is meant that preferably 0 to 100 nucleotides, more preferably 0 to 10 nucleotides, and most preferably 0 to 3 nucleotides exist between the 3' end of the primer and the point of deviation between the related nucleotides.
20 Figure 1 shows 0 nucleotides between primer and the point of deviation between the wild-type (Leu) and mutant (Pro) polynucleotides. Polymerization from such a proximal location will result in an extension product, the length of which will depend on the choice of dNTPs
25 and optional chain terminating, base pairing entities.

To conduct a simultaneous analysis of related polynucleotides, the hybridized primer is preferably extended by a polymerase in the presence of a nucleotide mixture of either i) two or three dNTPs and no chain-
30 terminating, base-pairing entities or ii) one to three dNTPs and one to three chain-terminating, base-pairing entities.

In the embodiment where mutant and wild-type sequences are to be extended in the presence of dNTP(s)
35 and ddNTP(s), the choice of the dNTP/ddNTP combinations is determined such that polymerase-catalyzed extension gives rise to short extension products of differing

length for wild-type and mutant targets. The choice of the particular chain terminating dideoxynucleotide(s) is decided by its complementarity to the suspected mutant nucleotide or to a nucleotide just after the mutant
5 sequence, if it allows for greater clarity in distinguishing between the mutant and the wild-type sequence.

Synthesis of the extension products is accomplished by polymerase extension of the primers until a template
10 nucleotide is read or omitted which terminates synthesis. For example, a nucleotide in the template can be read for which no complementary dNTP is available in the extension mixture, resulting in chain termination. Or, more preferably, a nucleotide in the
15 template can be read for which a complementary chain terminating, base pairing entity is available, likewise resulting in chain termination.

Various types of polymerases are useful for the primer extension step in the mutational analysis and
20 include, for example, DNA-directed and RNA-directed DNA polymerases. The type of polymerase depends on whether the nucleic acid suspected of containing a genetic mutation is either DNA or RNA. For example, if the nucleic acid is obtained by PCR amplification of a
25 subject's genetic material, then the nucleic acid is comprised of DNA. It may be desirable to directly analyze the mRNA or precursors thereof, or products of a transcription-mediated amplification systems such as the self sustained sequence replication (35R) reaction. For
30 such cases an RNA-directed DNA polymerase such as reverse transcriptase or rTth DNA polymerase is useful for the primer extension reaction. One skilled in the art can determine which, if any, of the possible polymerases is more or less beneficial to suit a
35 particular need or outcome.

To ensure accurate and reliable incorporation of the appropriate deoxynucleotide(s) and chain terminating

dideoxynucleotides, it is advantageous to perform the extension reactions with a high fidelity polymerase. The term "high fidelity" when used in reference to a polymerase is intended to mean those polymerases which exhibit 3'-5' exonuclease activity and concomitant proof-reading function. DNA polymerases are available that provide a proof-reading exonuclease activity which edits the nascent strand in a 3' to 5' (3'-5') direction to substantially reduce the number of incorporation errors. Specific examples of a high fidelity polymerase are, for example, *E. coli* DNA polymerase, Klenow fragment, T4 DNA polymerase, Vent[®], Pfu and ULTma[™] DNA polymerases. Other high fidelity polymerases exist as well and are known to those skilled in the art. Vent[®], Pfu and ULTma[™] DNA polymerases are particularly advantageous for the methods described herein because these are thermostable polymerases with 3'-5' exonuclease activity. Other thermostable polymerases include, for example, Taq, Vent[®] (exo⁻), Thermozyme[™], Exo⁻ Pfu and rTth DNA polymerases. One skilled in the art can determine which, if any, of the possible polymerases will be more or less beneficial to suit a particular need or outcome.

The ULTma[™], Vent[®] and Pfu polymerases also have thermostable properties in addition to their high fidelity properties. Thermostable polymerases offer additional advantages in that they are useful in automated thermocycling procedures to ensure complete transformation of primers to their extension products. Thus, the invention preferably provides increased reaction efficiency and detection sensitivity by repeating the primer extension step one or more times, by thermocycling the reaction and by using a thermostable polymerase.

Once the extension products have been created, they are analyzed. Comparison of the extension products derived from related polynucleotides such as wild-type

and mutant sequences are performed by a variety of methods well known to those skilled in the art. For example, the resolution of differentially extended primers is readily carried out using products that
5 distinguish nucleotide sequences on the basis of their size such as polyacrylamide gel electrophoresis, capillary electrophoresis or mass spectroscopy. As an example, directly determining the molecular weight of the extension products allows for the identification of
10 a mutant sequence. Alternatively, a simple comparison of relative molecular weights of the extension products derived from the mutant and wild-type sequences using the primer as a molecular weight standard also achieves the same results.

15 In many instances (for example, when using gel electrophoresis), it is desirable to use chemical moieties or physical structures that allow for rapid and efficient detection of the primer extension products. Such detection moieties include fluorescent labels,
20 radioisotope labels, biotin conjugates and the like. Fluorescent labels provide the advantage of non-isotopic detection, high sensitivity and automation for detection of the extension products. Where the various detection moieties described are attached to the primer, they are
25 either directly attached to the primer or indirectly attached to the primer by use of a linker-type molecule. Alternatively, in some applications, it is contemplated that the nucleotides used for extension or chain termination include detection moieties that are
30 incorporated into the product during polymerization and allow for subsequent detection.

The methods described above for simultaneously analyzing a single group of related polynucleotides, such as related wildtype and mutant sequences at the
35 same locus within a gene, also are useful to simultaneously analyze more than one group of related polynucleotides, such as multiple groups of related

wildtype and mutant sequences found within multiple genes or at different loci within the same gene. Such approaches are referred to herein as "multiplexing." An advantage of multiplexing is that the complexity of
5 analyzing multiple groups of related polynucleotides and the number of samples which is required to be handled is reduced.

In an embodiment of the invention, primers and nucleotide mixtures that are useful together within a
10 single reaction are chosen. In particular, primers of different sizes are selected to analyze multiple groups of related polynucleotides. Also, a nucleotide mixture is selected that will generate extension products of different length for each of the related
15 polynucleotides. In such situations, addition of the related polynucleotides to the nucleotide mixture in conjunction with the primers allows for primer extension and analysis of extension products in a similar fashion to that described previously. An example of this
20 embodiment is discussed below in connection with Figures 4A and 4B for the simultaneous interrogation of mutations at COX2 codons 20 and 95.

In another embodiment of the present invention, individual extension products are combined prior to
25 analysis. For example, the individual extension products are combined prior to electrophoresis and loaded in the same lane, thereby increasing sample throughput. As in the previous embodiment, primers used to interrogate different groups of related polynucleotides
30 should be of different lengths to ensure resolution of extension products. Unlike the previous embodiment, the nucleotide mixtures of the individual extension reactions may be different. In some instances, it may be necessary to use more than one nucleotide mixture to
35 generate a different size extension product for each polynucleotide interrogated.

In yet another embodiment of the invention, the gel used for resolution of the primer extension reaction products is loaded repeatedly by interrupting electrophoresis at various time intervals such that each lane contains several individual reaction products. This approach provides an effective way of increasing assay efficiency and throughput. The use of multiple loadings (e.g., of a conventional automated DNA Sequencer) make it possible to analyze multiple genes in a single gel lane. An example of this embodiment is discussed below in connection with Figure 8.

In still another embodiment of the invention, primers are labeled with different reporter molecules. Suitable non-isotopic labels include fluorophors which have different excitation/emission maxima. The use of different fluorophor labels permits independent detection of sets of extension products. This approach enables analysis of multiple extension reactions which contain primers of the same length but tagged with different fluorophors.

Various aspects and advantages of the present invention are observed in the embodiments associated with the Figures. Turning now to Figure 1, the methods of the invention are utilized to detect the presence of a mutation in one codon of one gene, for example, the cytochrome c oxidase (COX) gene. Mutation(s) in the mitochondrial-encoded subunit(s) of this gene correlate with the sporadic form of Alzheimer's disease (AD). The numbering scheme for the subunits I to III of the COX gene are based on Anderson et al., Nature, 290:457-465 (1981).

In particular, the cytochrome c oxidase subunit 2 (COX2) gene is amplified by PCR from DNA samples obtained from Alzheimer's patients and control patients in order to determine the sequence at codon 95, a susceptible mutation site in AD. Codon 95 in the wild-type COX2 gene codes for leucine (CTT), but in a certain

number of AD patients, a point mutation results in a codon change to proline (CCT) or phenylalanine (TTT). Figure 1 shows the wild-type leucine sequence (CTT codon) at the top of the diagram along with the two
5 known mutant sequences (middle and bottom). A primer having dA as its 3' nucleotide and which is complementary to the third nucleotide of the codon is useful to discriminate between wild-type (Leu) and mutant (Pro, Phe) sequences at codon 95. The choice of
10 ddGTP and dATP ensures that the primer is extended by two bases (dA and ddG), one base (ddG) and three bases (dA, dA and ddG), respectively, for the wild-type sequence, the proline mutant and the phenylalanine mutant. Thus, in the specific example shown
15 schematically in Figure 1, the Pro mutant is one nucleotide shorter and the Phe mutant is one nucleotide longer than the wild-type sequence. Separation of wild-type and mutant primer extension products of codon 95 by gel electrophoresis is shown in Figures 5A-5C.

20 Another embodiment of the invention provides a method for quantifying the level of heteroplasmy of a genetic mutation within a sample. Such a quantification is shown in Figure 2, where between 1% and 10% of the mutant sequence is mixed with the wild type sequence and
25 then assayed simultaneously with the sample suspected of containing the genetic mutation. The nucleotide combinations are designed such that the extension products derived from the wild-type templates are one base longer than those derived from the mutant
30 templates. For each codon the lanes are represented as follows: Lane 1: wild-type; lane 2: mutant; lane 3: 10% mutant; lane 4: 5% mutant; lane 5: 1% mutant.

As noted above, mutations in more than one codon of the same gene may be analyzed simultaneously by the
35 method of this invention. Two conditions are preferably fulfilled to complete this multiplexing strategy: (a) primers corresponding to different codons are preferably

of different lengths so that extension products for each codon may be separated by size, and (b) in the event dNTP/ddNTP mixtures are used in primer extension of multiple codons, such mixtures yield reaction products which permit discrimination of wild-type and mutant targets for each of the interrogated codons. As also noted above, there are at least two alternatives for simultaneously analyzing multiple mutations. The first alternative is to perform reactions that have compatible primer/nucleotide combinations in a single tube. The second alternative is to pool different reactions prior to gel loading. The example provided in Figure 3 shows a set of three multiplexed reactions for both the COX1 and COX2 targets. These are loaded on two lanes at three different time points, allowing a total of 14 codons to be analyzed.

Analysis of multiple mutations in a single reaction is also described in connection with Figures 4A and 4B. The detection of mutations at codons 20 and 95 of the COX2 gene in a single reaction using the appropriate primer/nucleotide combination is provided in these Figures. Schematics of the primer/nucleotide combination for COX2 codon 20 (Figure 4A) and COX2 codon 95 (Figure 4B) are shown. The primers for COX2, codon 20 and COX1, codon 95 are designed to be 24 and 20 nucleotides in length, respectively. Analysis of COX2, codon 20, using the nucleotide combination dATP and ddGTP generates primer extension products from the mutant (Pro) and wild-type (Leu) DNA templates that are 25 and 26 nucleotides in length, respectively. The same nucleotide combination for COX2, codon 95, produces primer extension products from the mutant (Pro) and wild-type (Leu) DNA templates that are 21 and 22 nucleotides in length, respectively.

Since single base resolution of nucleic acids which are smaller than 100 nucleotides in length is easily achieved by gel electrophoretic techniques, primer

extension reactions for COX2, codon 20 and COX2, codon 95 are loaded in the same gel lane. Therefore, addition of both primers to a reaction mixture that contains a DNA template which spans both codons and contains a dATP/ddGTP nucleotide mixture permits simultaneous analysis of codons 20 and 95, as exhibited in Figures 5A-5C.

Figure 5A uses Vent[®] DNA polymerase with dATP and ddGTP for analyzing COX2, codons 20 and 95. Lane 1: codon 20 primer (primer 20), no template; lane 2: primer 20, wild-type (wt) template; lane 3: primer 20, mutant codon 20 template (mutant 20); lane 4: codon 95 primer (primer 95), no template; lane 5: primer 95, wild-type template; lane 6: primer 95, mutant codon 95 template (mutant 95); lane 7: primers 20 and 95, no template; lane 8: primers 20 and 95, wild-type template; lane 9: primers 20 and 95, mutant 95.

Figure 5B shows the use of Taq DNA polymerase with dATP and ddGTP in the primer extensions for analysis of COX2 codons 20 and 95. Lane 1: primer 95, no template; lane 2: primer 95, wild-type template; lane 3: primer 95, mutant 95; lane 4: primer 20, no template; lane 5: primer 20, wild-type template; lane 6: primer 20, mutant 20; lane 7: primers 20 and 95, wild-type template; lane 8: primers 20 and 95, mutant 95.

Figure 5C shows the use of ULTma[™] DNA polymerase with dATP and ddGTP in the primer extensions for analysis of COX2, codons 20 and 95. Lane 1: primer 95, no template; lane 2: primer 95, wild-type template; lane 3: primer 95, mutant 95; lane 4: primers 20 and 95, no template; lane 5: primers 20 and 95, wild-type template; lane 6: primers 20 and 95, mutant 20.

Figure 6 illustrates the interrogation of the 3460 mutation of the ND1 mitochondrial gene. A point mutation at this site results in a codon change from alanine (GCC) to threonine (ACC) at amino acid position 52 of the gene product. Specifically, in six

independent pedigrees, a substitution of threonine for alanine at position 52 of the ND1 protein (nucleotide 3460) is shown to correlate with the development of Leber Hereditary Optic Neuropathy (LHON). These studies are described by Howell et. al., Am. J. Hum. Genet., 49: 939-950 (1991), which is herein incorporated by reference. The pedigrees described by Howell et al. are used for the primer extension and sequencing comparisons. The primer is designed to have dG as its 3' base which is complementary to the second nucleotide of the codon. Two different dNTP/ddNTP combinations are used to independently assess the presence of the mutation. In the first case, dCTP is used with ddGTP and ddTTP. Addition of dC followed by chain termination with ddG provides an extension product from the wild-type sequence which is two bases longer than the primer. The extension product of the threonine mutant is simply the addition of a chain terminating ddT residue. The use of the second nucleotide combination (dTTP, ddGTP, ddCTP) provides the converse product size mixture. The wild-type sequence directs the addition of ddC, whereas the mutant sequence directs the addition of dT and ddG. Using either combination, the presence of the mutation is easily deduced based on the sizes of the product relative to the primer.

Figure 7 illustrates a typical standard curve useful for quantifying the heteroplasmy of an AD-associated mutation at codon 71 of the COX2 gene. The % mutant as detected by the primer extension method of the present invention is plotted against the actual % mutant in the target sample.

Figure 8 illustrates the results of a mutation analysis by gel electrophoresis of codon 415 of the COX1 gene for 60 patient samples using multiple sample loadings. Loadings 1 and 2 are for reactions providing longer wild type template-derived product and loadings 3 and 4 are for reactions providing longer mutant

template-derived product. Loading 1: lane 1, primer, no
template; lane 2, wildtype control; lane 3, mutant
control; lanes 4-33, samples 1-30. Loading 2: lane 1,
primer, no template; lane 2, wildtype control; lane 3,
5 mutant control; lanes 4-33, samples 31-60. Loading 3:
lane 1, primer, no template; lane 2, wildtype control;
lane 3, mutant control; lanes 4-33, samples 1-30.
Loading 4: lane 1, primer, no template; lane 2, wildtype
control; lane 3, mutant control; lanes 4-33, samples 31-
10 60. The analysis shows that samples 2-4 have a
homoplasmic mutation at codon 415.

The foregoing and following description of the
invention and the various embodiments are not intended
to be limiting of the invention but rather is
15 illustrative thereof. Those skilled in the art of
molecular genetics can formulate further embodiments
encompassed within the scope of the present invention.

EXAMPLE I

20 Analysis of Mutations within the Mitochondrial Gene Encoding Cytochrome C Oxidase

This example illustrates the use of multiplex
primer extension to diagnose mutations in cytochrome c
25 oxidase genes (COX). The format employs single primers
and individual reactions to diagnose mutations at codons
20 and 90 of the COX2 gene.

For sample amplification, PCR primers are
synthesized which flank codons 20 and 90 of the COX2
30 gene. The primer sequences synthesized for the COX2
gene are as follows: COX2 (sense):
5'-CAAGCCAACCCCATGGCCTCC-3' (SEQ ID NO: 1); COX2
(antisense) 5'-AGTATTTAGTTGGGGCATTTCAC-3' (SEQ ID NO:
2); and COX2 (antisense) 5'-GACGTCCGGAATTGCATCTGTTTT-3'
35 (SEQ ID NO: 3).

Oligonucleotides are synthesized on an ABI 394
DNA/RNA synthesizer using phosphoramidite chemistry as

recommended by the manufacturer (Perkin Elmer, Applied Biosystems Division, Foster City, CA). Various labels are incorporated into the oligonucleotide primers, using methods known in the art and conditions recommended by the manufacturers. For example, fluorescein-labeled oligonucleotides are obtained by using Fluordite reagent (Millipore, Malborough, MA) or FAM Amidite (Perkin Elmer) in the last step of automated synthesis. Synthesis of biotin-labeled oligonucleotides is carried out by using Biotin Amidite reagent (Perkin Elmer) in the last step of automated synthesis. The oligonucleotide primers are purified by reverse phase chromatography using an acetonitrile gradient in 0.1 M triethylammonium acetate, pH 6.8, running buffer. The purified oligonucleotides migrate as single bands on a 15% denaturing polyacrylamide gel.

The mitochondrial DNA of patients diagnosed to have Alzheimer's disease is extracted by the following method. Briefly, 7-8 ml samples of blood are collected from each patient in EDTA Vacutainer tubes (Scientific Products, Waukegan Park, IL). Six ml of each blood sample is transferred to a 15 ml polypropylene tube and frozen at -80°C for 30 min. The sample is thawed at 37°C and then placed on ice. An equal volume of cold 10 mM EDTA, pH 8.0, 10 mM NaCl is added and mixed by inverting the tube. Following incubation in ice for an additional 5 minutes, the sample is centrifuged at 5000 rpm for 10 minutes. The supernatant is aspirated off and 5 ml of 10 mM EDTA, pH 8.0, 10 mM NaCl is added to the pellet for a further wash. Gentle agitation is used to resuspend the pellet and the mixture is again centrifuged at 5000 rpm for 10 min. The supernatant is removed and the pellet resuspended in 3 ml of Lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.2% SDS, 200 µg/ml Proteinase K). To lyse the cells and digest cellular proteins, the mixture is vortexed vigorously until a clear solution is obtained and then

incubated at 50°C for 45 min, with a 10-15 s vortex every 10 min.

DNA is isolated from the above samples by ethanol precipitation following phenol/chloroform extraction.

- 5 Briefly, 3 ml of phenol:chloroform:isoamyl alcohol (50:48:2) is added to each sample and the mixture vortexed, followed by centrifugation at 5000 rpm for 2 min to separate the phases. The aqueous phase is carefully withdrawn after the last centrifugation step.
- 10 DNA is precipitated by mixing each sample with 300 μ l of 3 M NaOAc and 6 ml of 100% ethanol. The genomic DNA is precipitated in a dry ice/ethanol bath at -20°C overnight and then pelleted by centrifugation at 17000 g for 20 min at 4°C. The pellet is washed with 70%
- 15 ethanol and resuspended in 50-400 μ l of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and DNA quantitated by A_{260} absorbance of a 1:50 dilution.

- An alternative procedure also useful for small scale DNA extraction from whole blood samples of
- 20 patients is as follows. A 50 μ l aliquot of whole blood is mixed with 500 μ l volumes of TE and then resuspended in 100 μ l of a buffer containing 50 mM KCl, 10 mM Tris.Cl pH 8.3, 1.5 mM $MgCl_2$, 0.5% Tween 20 and 100 μ g/ml Proteinase K. The mixture is incubated for 45 min at
- 25 56°C, and then the protease is heat-inactivated by incubation at 95°C for 10 min. Ten μ l of the final solution is used for PCR amplification. Typically, whole blood contains 5000 white blood cells/ μ l, so the DNA extracted is estimated to be from approximately
- 30 250,000 nucleated cells.

- COX2 gene fragments are obtained from the DNA preparations described above by PCR amplification. PCR reactions are performed in 50 μ l final volume which contained 100-1000 ng of DNA, 2.5 U of AmpliTaq[®] DNA
- 35 polymerase (Roche Molecular Systems, Branchburg, New Jersey), 20 pmol of each primer (SEQ ID NO: 1 and SEQ ID NO: 3) and 10 nmol of each dNTP in PCR buffer (10 mM

Tris.HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂). After initial denaturation at 95°C for 10 s in a Gene Amp PCR System 9600 thermal cycler (Perkin Elmer, Norwalk CT), the samples are amplified for 25 cycles under the following conditions: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 4 min.

To generate biotin-labeled PCR products, the PCR amplifications are performed with 4.5 pmol of biotin-labeled sense primer (SEQ ID NO: 1) and 15 pmol of antisense strand primer (SEQ ID NO: 2). The PCR conditions for the biotin-labeled primers are an initial denaturation at 95°C for 10 s followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension at 72°C for 7 min. All PCR products are analyzed by electrophoresis on a 0.8% agarose gel (Sea Kem LE Agarose, FMC Corporation) to ensure that the appropriate size fragment was amplified.

Calf intestine alkaline phosphatase (1 unit Boehringer Mannheim, Indianapolis, IN) in 5 µl of buffer containing 10 mM MgCl₂, 10 mM ZnCl₂, 100 mM Tris-HCl, pH 8.3 is added to each PCR reaction mixture, and the reaction tubes placed in a Gene Amp PCR System for 9600 cycles for 30 min at 37°C. Then 1.1 µl of 0.25 M EDTA, pH 8.00 is added, and the alkaline phosphatase denatured at 75°C for 10 min. Following denaturation, double-stranded PCR products are purified away from the primers, nucleotides and the enzymes using QIAQuick® (Qiagen, Chatsworth, CA) columns following procedures recommended by the manufacturer. Briefly, the 5 volumes of QIAQuick® buffer PB are added to 1 volume of the PCR reaction and mixed. A Qiaquick spin column is placed in a 2 ml collection tube followed by transfer of the PCR reaction mixtures to the column. The column is centrifuged for 2 min at 14000 g, and the flowthrough is discarded. The absorbed PCR product is washed with one 750 µl volume of QIAQuick® buffer PE, and then eluted with 50 µl of 10 mM Tris-HCl, pH 8.5. The purified

product solution is dried in a Savant SpeedVac Concentrator (Savant Instruments, Inc., Farmingdale, NY) and then reconstituted in 20 μ l of water.

Biotin-labeled products are purified from the reactions using streptavidin-coated magnetic beads (Dynabeads[®] M-280 Streptavidin; Dynal, Inc. Lake Success, NY). Briefly, a 20 μ l suspension of Dynabeads[®] M-280 Streptavidin in a 1.5 ml microfuge tube is placed in a Dynal MPC[®]-E-1 magnet and the supernatant is removed. The beads are washed with 20 μ l of TTL buffer (6 M LiCl, 300 mM Tris-HCl, pH 8.0, 0.3% Tween-20) and then resuspended in 20 μ l of TTL buffer, followed by the addition of 40 μ l of a biotin-labeled PCR product mixture. The mixture is incubated at 48°C for 30 min and the beads isolated by magnetic separation. The immobilized double-stranded DNA is washed three times with 100 μ l of TT buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween-20), taken up in 20 μ l of water and stored at 4°C. Multiple samples are processed in a microtitre-plate format using the Dynal MPC[®]-9600 magnetic particle concentrator.

For mutational analysis, primer extension reactions are performed as follows. Briefly, stock solutions of each dNTP and ddNTP are prepared by mixing equimolar amounts of the nucleotides (United States Biochemical Corporation, Cleveland, OH), with MgCl₂ and diluting the mixture to the desired concentration with TE. Fluorescein-labeled primers are diluted in TE to provide final stock concentrations of 40 fmol/ μ l. An 8X concentration of the final reaction buffer is prepared for the Taq DNA polymerase assays. The 10X reaction buffers for the Vent[®] (exo⁻) DNA polymerase and Exo⁻ Pfu DNA polymerase are used as supplied by the respective manufacturers. The 8X or 10X buffers are aliquoted such that, after addition of all other components, the final concentration of the buffer is 1X in the reaction mixture (see hereinbelow for final buffer

concentrations for various enzymes). All primer extension reactions are carried out in a final volume of 8 μ l. Master mixes of the appropriate dNTP/ddNTP/primers combinations are prepared such that

5 4.5 μ l of the required mix is dispensed into each reaction tube. The enzyme master mixes, supplemented as needed with MgCl₂ or DMSO, are prepared such that 2.5 μ l is aliquoted for each reaction. One μ l of the Qiagen-PCR amplified DNA (~100-500 fmol) is used as template

10 for the assays. After initial denaturation at 95°C for 2 min in a Gene Amp PCR System 9600 thermal cycler, the primer extension reaction conditions consist of 20 cycles of 95°C for 20 s and 55°C for 40 s. The samples are then concentrated to ~1 μ l by incubating the

15 reaction tubes at 94°C for 7 min followed by addition of 3 μ l of loading dye (0.2% blue dextran in 99:1 formamide/H₂O) to each sample. Prior to gel electrophoretic analysis of the reaction products, the tubes are stored at -20°C. Primer extension reactions

20 are performed with various polymerases, as described hereinbelow.

Primer extension reactions with ULTma™ DNA polymerase (Perkin Elmer, Norwalk, CT) containing 25 μ M and 400 μ M concentrations of dNTPs and ddNTPs,

25 respectively, 20 pmol fluorescein-labeled primer and 0.6 U of enzyme in ULTma™ reaction buffer [10 mM Tris-HCl, pH 8.8, 2 mM MgCl₂, 10 mM KCl and 0.002% Tween 20] are conducted.

For primer extension with Vent® (exo-) DNA

30 polymerase (New England Biolabs), the reactions contain 50 μ M and 400 μ M concentrations of dNTPs and ddNTPs, respectively, 20 pmol fluorescein-labeled primer and 1.5 U of enzyme in Vent® reaction buffer [20 mM Tris.HCl, pH 8.8, 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton

35 X-100].

Tag DNA Polymerase-catalyzed primer extension reactions contain the template with the appropriate

dNTP/ddNTP combination (dNTPs: 25 μ M; ddGTP, 25 μ M; ddCTP, 125 μ M; ddATP, 250 μ M; ddTTP, 500 μ M), 20 pmol fluorescein-labeled primer and 0.2 U of enzyme in Taq reaction buffer (15 mM Tris-HCl, pH 8.8, 2.5 mM MgCl₂, 50 mM KCl and 5% DMSO).

One unit of Exo⁻ Pfu DNA polymerase (Stratagene, Inc., San Diego, CA), Tth DNA polymerase and Thermozyme DNA polymerase (United States Biochemical Corporation, Cleveland, OH) is added for the respective primer extension reactions using these enzymes. The primer and nucleotide concentrations are identical to those used for ULTmaTM DNA polymerase.

For each of the primer extensions described above, the reaction products are either analyzed on a Millipore Base Station DNA Sequencer (Millipore Corporation, Bedford, MA) or an ABI 373 DNA Sequencer (Perkin Elmer). 10% and 12% denaturing polyacrylamide gels are used in the Millipore System and ABI system, respectively, with Tris-borate/EDTA as running buffer. During the gel pre-electrophoresis stage, the samples in loading dye are denatured for 3 min at 85°C. Three μ l aliquots of each primer extension reaction mixture are then loaded and electrophoresed according to the manufacturer's instructions. The mobility pattern of the extended primers are recorded by photoimaging the screen display.

Quantitative heteroplasmy analysis is, for example, carried out by interfacing with the Bio Image[®] Whole Band Analyzer software program (Bio Image Inc., Ann Arbor, MI). Quantitative heteroplasmy analysis in the ABI sequencing system is obtained by using the GENESCANTM 672 software for analyzing the fluorescent electrophoretograms. For routine quantification of heteroplasmy, it is useful to construct a standard curve, such as that shown in Figure 7 for an AD-associated mutation at codon 71 of the COX2 Gene. To generate Figure 7, plasmid DNA (10^{-17} mol) containing mutant and wildtype plasmid mixtures (corresponding to a

mutation site at COX2: codon 71) are amplified by PCR using the same primers as in Example I and aliquots of the purified PCR products are used in ULTma™ DNA polymerase-catalysed primer extension reactions using
5 5'-AACTATCCTGCCCCGCA-3' (SEQ. ID. NO: 4) as a primer with a nucleotide mixture containing dT and ddC. The primer extension reaction products are electrophoresed in an ABI 373 DNA sequencer and the electrophoretogram is analyzed by GENESCAN™ 672
10 software. The heteroplasmy detected for each mixture is plotted against % mutant plasmid.

The results obtained using the different polymerases show significant extension runthrough past the terminating ddNTP base when DNA polymerases lacking
15 3'-5' exonuclease activity are used (i.e. Taq, Tth, Exo⁺, Pfu, Vent⁺ (exo⁻) and Thermozyme). Although not wanting to be limited by theory, the most likely cause is base misincorporation by these enzymes (see Nucl. Acids Res., 20: 4567-45783, (1992) for Taq polymerase study). In
20 addition, the 5'-3' exonuclease activity of Taq polymerase is observed to cleave the fluorescein-labeled primers when greater than 0.2 U of the enzyme is used in the reactions.

One way to rectify the runthrough problem is by
25 using high fidelity polymerases such as ULTma™ DNA polymerase, since its 3'-5' exo activity is able to edit base mismatches. Figure 2 shows the results of a heteroplasmy analysis using an ULTma™ DNA polymerase-catalyzed primer extension reaction. The results shown
30 in Figure 2 are obtained from an analysis which is used to interrogate COX2 codons 20 and 90 of a sample containing either 1, 5 or 10% of mutant plasmid DNA in a wild-type plasmid background. Briefly, ten amol (10^{-17} moles) amounts of the above mixtures, which approximate
35 the amount of mitochondrial DNA present in 100 ng blood-extracted DNA, are amplified by PCR prior to the primer extension reaction. The extension primers are labeled

with fluorescein at the 5' termini and have the following sequences: COX2:20

5'-AGGGCGTGATCATGAAAGGTGATA-3' (SEQ. ID. NO: 5);

COX2:90, 5'-CGCATCCTTTACATAACAGACGAG-3' (SEQ ID NO: 6).

- 5 The nucleotide mixture for the codon 20 extension are dA and ddG, while the nucleotide mixture for the codon 90 extension are dG, ddA, and ddT. Lane 1: wildtype, lane 2: mutant; lane 3: 10% mutant; lane 4, 5% mutant; lane 5, 1% mutant. As shown in Figure 2, the assay is
- 10 clearly able to detect 5% heteroplasmy for codon 20 and 1% heteroplasmy for codon 90.

Various combinations of primers and nucleotide mixtures for integrating mutations at codons 155, 167, 178, 193, 194 and 415 of COX1 and codons 20, 22, 68, 71,

15 74, 90, 95, 110 and 146 of COX2 are found in Table I.

- Table I illustrates various primer sequences that are used for interrogating the AD-associated codon sites in COX1 and COX2 genes. The primers are shown with their respective nucleotide mixtures for extension.
- 20 Each codon site is shown to be monitored by two independent reactions using the same oligonucleotide primer. In Table IA, the nucleotide combinations are designed such that the wildtype templates direct synthesis of extended primer products which are longer
- 25 than those derived from the mutant template. In Table IB, the converse is true. All the primer extension reactions are carried out using ULTma™ DNA polymerase according to the procedure described in Example III below.

TABLE IA

Gene	Mutation (Codon No)	Primer and nucleotide combinations for AD-associated mutations					Nucleotide Mixture ¹						
		Longer Wild-Type Product	Primer Sequence	SEQ ID NO	Codon change (w/m)	Sense	Primer length bases	T _m °C	Product lengths bases	GA	GC	GG	AT
COX1	155	5'-TGGCCCTTAGATAGGAGA-3'		7	Val/Ile	-	21	55	w21, m22	X			X
COX1	167	5'-GCGGGGGTTTATATCATATTG-3'		8	Thr/Ala	-	25	57	w28, m26			X	
COX1	178	5'-CGAAGAGCGCGTTGGTAT-3'		9	Gln/Leu	-	20	58	w22, m21		X		
COX1	193	5'-GACTGGAGATAGGAGAGTACG-3'		10	Val/Ala/Ile	-	25	55	w28, m26 & m27	X	X		X
COX1	194	5'-AGGACTGGAGATAGGAGAGTAA-3'		11	Leu/Phe	-	25	55	w28, m26			X	
COX1	415	5'-ACTAGCGCAATCCATTTC-3'		12	Thr/Ala	+	21	55	w23, m22	X		X	
COX2	20	5'-AGGCGGTATCATGAAGGTGTA-3'		5	Leu/Pro	-	24	62	w26, m25	X			X
COX2	22	5'-TCCCTATCATAGAGGCTTATCA-3'		13	Thr/Ile	+	25	55	w28, m26		X		
COX2	68	5'-GACTAGATGATGCGGCCA-3'		14	Leu/Phe	-	20	60	w23, m21		X		
COX2	71	5'-AAGTATCTGCGGCCCA-3'		4	Ile/Thr	+	17	55	w19, m18			X	
COX2	74	5'-GGGAGCGGATGAGGA-3'		15	Val/Ala	-	16	55	w18, m17		X		
COX2	90	5'-GCGATCTTTACATACAGACGAG-3'		6	Val/Ile	+	24	57	w26, m25			X	
COX2	95 (a)	5'-GGCCAAATGATTGTGTTA-3'		16	Leu/Pro/Phe	-	20	53	w22, m21 & m23	X			X
COX2	95 (b)	5'-GGCCAAATGATTGTGTTA-3'		17	Leu/Ile	-	21	53	w25, m22		X		
COX2	95 (c)	5'-GGCCAAATGATTGTGTTA-3'		17	Leu/Phe	-	21	53	w25, m22		X		
COX2	110	5'-CACCAGTGTACTGACCTACGAG-3'		18	Tyr/His/Cys	+	24	57	w27, m26 & m25	X		X	
COX2	146	5'-ATTATTACGAATGGGGCTTCA-3'		19	Ile/Val/Thr	-	24	57	w27, m26 & m25	X		X	X

¹ For brevity, all dNTP's are abbreviated as dN's, and all ddNTP's are abbreviated as ddN's in this Table.

² T_m (melting temperature) is the temperature at which 50% of the primer is annealed to its complementary template.

TABLE IB

		Primer and nucleotide combinations for AD-associated mutations										Nucleotide Mixture					
Gene	Mutation (Codon No)	Longer Mutant Product	Primer Sequence	Primer ID No	Codon change (w/m) (w/m/m)	Sense	Primer Length bases	Tm °C	Product Length bases	dA	dC	dG	dT	dAA	dAC	dAG	ddT
COX1	155	5'-TGGCCCTAGATAGAGAGGA-3'		7	Val/Ile	-	21	55	w21,m23				X	X	X		
COX1	167	5'-GCAGGCGTTTATATGATTAATG-3'		8	Thr/Ala	-	25	57	w26,m27		X						X
COX1	178	5'-CGAGAGAGGCGTTTGGTAT-3'		9	Gln/Leu	-	20	58	w21,m22	X						X	X
COX1	193	5'-CTGATCCCTCTAATACAGCA-3'		20	Val/Ala Ile	+	22	57	w25,m24 w23			X	X	X	X		
COX1	194	5'-AGGACTGGGAGGATAGAGAGTA-3'		11	Leu/Phe	-	25	55	w26,m27	X						X	
COX1	415	5'-ACCTACCGCAAAATCCATTTC-3'		12	Thr/Ala	+	21	55	w22,m23			X		X	X		
COX2	20	5'-AGGCGCTGATCATGAAAGGTGATA-3'		5	Leu/Pro	-	24	62	w25,m27			X		X	X		
COX2	22	5'-TCCCTTATCTAGAGAGGCTTATCA-3'		13	Thr/Ile	+	25	55	w26,m27				X		X		
COX2	68	5'-GACTAGGATGATGGCGGCA-3'		14	Leu/Phe	-	20	60	w21,m22	X						X	
COX2	71	5'-AACTATCTGCGCCGCCA-3'		4	Ile/Thr	+	17	55	w18,m20		X			X	X		X
COX2	74	5'-GGGAGGCGATGAGCA-3'		15		-	16	55	w17,m18				X	X	X		
COX2	90	5'-CCCATCTTTACNTAACGACGACG-3'		6	Val/Ile	+	24	57	w25,m26	X						X	X
COX2	95 (a)	5'-GGCCAAATTTGATTTGATGTA-3'		16	Leu/Pro	-	20	53	w21,m25			X		X			
COX2	95 (b)	5'-GCCCAATTTGATTTGATGTA-3'		17	Leu/Ile	-	21	53	w22,m23				X			X	
COX2	110	5'-CACCAATGGTACTGAACCTACGAG-3'		18	Tyr/His Cys	+	24	57	w26,m27 w25			X	X	X	X		
COX2	146	5'-ATTATATACGAATGGGGGCTTCA-3'		19	Ile/Val Thr	-	24	57	w26,m28 w25	X	X					X	X

The efficiency of the method of the present invention is further increased through multiple sample loadings.

Figure 8 illustrates mutation analysis by gel electrophoresis of codon 415 of the COX1 gene for 60 patient samples using multiple sample loadings. Cellular DNA is extracted from 60 individuals, and PCR amplification of a region encompassing codon 415 of the COX1 gene is carried out by using the primers 5'-CCATCATAGGAGGCTTCATTCAGT-3' (forward) (SEQ. ID. NO: 21) and 5'-TGATAGGATGTTTCATGTGGTGTATGC-3' (reverse) (SEQ. ID. NO: 22). The PCR products (200 bases in length) are purified as described in Example III and used as templates in primer extension reactions. The mutation site is analyzed by two independent primer extension reactions. In the first reaction the fluorescein-labelled primer (5'-ACCTACGCCAAAATCCATTTC-3') (SEQ. ID. NO: 12) is extended with dATP, ddCTP and ddGTP such that the extended primer from the wildtype template is longer than that derived from mutant template. The converse occurs when the primer is extended in the presence of dGTP, ddATP and ddCTP.

Loadings 1 and 2 are for reactions providing longer wild type template-derived product and loadings 3 and 4 are for reactions providing longer mutant template-derived product. Loading 1: lane 1, primer, no template; lane 2, wildtype control; lane 3, mutant control; lanes 4-33, samples 1-30. Loading 2: lane 1, primer, no template; lane 2, wildtype control; lane 3, mutant control; lanes 4-33, samples 31-60. Loading 3: lane 1, primer, no template; lane 2, wildtype control; lane 3, mutant control, lanes 4-33, samples 1-30. Loading 4: lane 1, primer, no template; lane 2, wildtype control; lane 3, mutant control; lanes 4-33, samples 31-60. The analysis shows that samples 2-4 have a homoplasmic mutation at codon 415.

EXAMPLE II

Simultaneous Analysis of Multiple Mutations within a Single Reaction

5 In addition to the results described above, other experiments show that the method of the present invention can be multiplexed to simultaneously interrogate two codons in the same reaction. Such multiplexed primer extension assays significantly reduce
10 the number of reactions required to analyze multiple mutations in a DNA sample. Since the assay is based on size resolution, it is easily automated on gel-based platforms. Software programs, such as GENESCAN™ 672 software, capable of quantitating electrophoretogram
15 fluorescence intensities permit accurate quantitation of low level heteroplasmy, a feature not available with other mutational analysis methods.

This example shows the simultaneous analysis of multiple mutations within a gene using a single primer
20 extension reaction.

Mutations at codons 20 and 95 of the COX2 mitochondrial gene are analyzed by multiplexed primer extension. Plasmid targets harboring the wild-type or mutant COX2 gene sequences are amplified by PCR and
25 interrogated for presence of mutations. The conditions for the reaction are similar to those in Example I. The primers and nucleotide mixtures are schematically shown in Figure 4. The primers are labeled with fluorescein at the 5'-termini and have the following sequences:
30 COX2:20, 5'-AGGGCGTGATCATGAAAGGTGATA-3' (SEQ ID NO: 5);
COX2:95, 5'-GGCCAATTGATTTGATGGTA-3' (SEQ ID NO: 16).
Briefly, extensions for the wild-type and mutant sequences of codon 20 result in products of 26 and 25 nucleotides in length, respectively (Figure 4A).
35 Extensions for codon 95 on the other hand result in products of 22 and 21 nucleotides, respectively (Figure 4B).

The results of the primer extension reactions analyzing both codons 20 and 95 in a single reaction are shown in Figure 5. The data shown in this figure are the result of three similar experiments, wherein the primer extensions are performed with a different thermostable DNA polymerase. For example, Figure 5A shows the analysis using Vent[®] (exo-) DNA polymerase. Figures 5B and 5C show a similar analysis to that in Figure 5A. However, these reactions substitute the Vent[®] (exo-) polymerase with Tag and Ultma[™] DNA polymerases, respectively.

In the analysis shown in Figure 5A, lanes 1-3 correspond to primer extension reactions analyzing codon 20 alone. Briefly, lane 1 contains the primer without template; lane 2 contains the primer with the wild-type template; whereas lane 3 contains the primer with the mutant template (codon 20) sequence. Lanes 4-6 essentially duplicate lanes 1-3 except that the primer for codon 95 is substituted for the codon 20 primer and the mutant template in lane 6 is that for codon 95. Lanes 7-9 show the analysis of both codons in a single reaction. Lane 7 corresponds to reactions performed in the presence of primers for both codons 20 and 95 in the absence of template. The products in this reaction correspond exactly to those independently observed in lane 1 and lane 4. Lane 8 corresponds to reactions performed using the wild-type template and both primers. Again, the extension products correspond exactly to those independently observed in lanes 2 or 5, showing that the sequence at each codon position is that of the wild-type. Lane 9 also analyzes both codon positions. However, one of the codons corresponds to a mutant sequence and one corresponds to the wild-type sequence. As is observed, the top band in this lane corresponds to the wild-type sequence at codon 20, whereas the bottom band reveals the presence of the mutant sequence at codon 95. These results demonstrate the accurate

analysis of multiple codons in a single reaction using the multiplex primer extension methods of the present invention.

The primer extensions shown in Figures 5B and 5C similarly show the analysis of multiple codons using identical methods. However, these reactions are performed using either *Taq* or *Ultma*TM polymerases, respectively. Briefly, Figure 5B, lanes 1-3 show extensions in the absence of template or in the presence of either wild-type or mutant templates for codon 95, respectively. Lanes 4-6 parallel lanes 1-3 except the primer and templates are for codon 20. Lane 7 assesses the sequence of codons 20 and 95 using a wild-type template while lane 8 assesses these codon sequences in a template where codon 95 is mutated.

Figure 5C also shows an analysis of multiple codons in a single reaction. The polymerase used for the extension reactions is *Ultma*TM DNA polymerase. Only six lanes are shown in this figure where the first three correspond to codon 95 analysis in the absence of template (lane 1) or presence of either wild-type (lane 2) or mutant template (lane 3). Lanes 4 through 6 are primer extensions with primers present for analysis of both codons 20 and 95. The first lane of this series does not contain template nucleic acid, whereas the second lane contains the wild-type template. The last lane contains a template which is wild-type for codon 95 and mutant for codon 20. The results shown in Figures 5B and 5C further corroborate those shown in Figure 5A using the *Vent*[®] (exo-) polymerase and demonstrate the accurate and simultaneous determination of the presence of multiple mutations in a single reaction.

EXAMPLE III

Comparison of Multiplex Primer Extension
and Direct Sequencing for Determining the Presence of
Genetic Mutations

5

This example assesses the accuracy of multiplex primer extension for detecting the presence of mutations within a sample population by directly comparing the results to that obtained by DNA sequencing.

10 The mutation used for comparison is that previously described for the 3460 mitochondrial ND1 protein. The DNA sequence analysis is carried out according to the protocol of Howell et al. Am. J. Hum. Genet., 49: 939-950 (1990). Briefly, genomic DNA is first isolated from
15 white blood cell/platelet fraction of blood samples. The mitochondrial complex 1 genes which include the ND1 gene are amplified by PCR using standard conditions as a series of 23 overlapping gene fragments. For DNA sequencing, a minimum of 20 clones is analyzed to
20 estimate the degrees of heteroplasmy. The results are presented below in Table II.

For the primer extension analysis, genomic DNA is amplified from the samples above using specific primers which flank the ND1 gene. The sequence of these primers
25 are as follows. ND1-1A: 5'-CAGTCAGAGGATCAATCCCTC-3' (SEQ ID NO: 23) and ND1-1B: 5'-GAGGGGGGATCATAGAAG-3' (SEQ ID NO: 24). PCR amplification is performed as described previously and the products are first purified by a two step procedure being used as a template for
30 primer extension. First, the products are treated with calf intestine alkaline phosphatase (Boehringer Mannheim) to dephosphorylate residual dNTPs for the amplification reaction (1U alkaline phosphatase/50 μ l PCR reaction containing 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 10 mM
35 Tris-HCl, pH 8.3). Secondly, the PCR products are then purified using the Qiagen QIAquick™ PCR purification kit.

The primer extension reactions is performed as previously described. Briefly, the reactions consist of 10 mM Tris-HCl, pH 8.8, 10 mM KCl, 0.002% Tween 20, 2 mM MgCl₂, 20 fmol fluorescein-labeled primer, 1 µl purified PCR product, 400 µM ddNTPs/25 µM dNTPs, 0.6 U Ultma™ DNA polymerase in a 8 µl total volume. The thermocycling consists of 2 minutes at 95°C followed by 20 cycles of 20 s at 95°C, 40 s at 55°C and an indefinite hold at 4°C when the cycles are completed.

10 To assay for the presence of the mutation at nucleotide 3460 (Ala52 to Thr52), two different primer/nucleotide combinations are used. The primer used for the extensions is labeled with fluorescein at the 5' terminal and has the sequence

15 5'-GCTCTTTGGTGAAGAGTTTATGG-3' (SEQ ID NO: 25) and its use with the two different nucleotide combinations is shown in Figure 6. Briefly, the nucleotide mixtures in this analysis consist of one nucleoside triphosphate and two chain terminating dideoxynucleoside triphosphates.

20 In the upper reaction, the nucleotide mixture yields a longer extension product from the wild-type template. Conversely, in the lower reaction, it is the mutant template that yields a longer extension product. Quantitation of the percentage of mutants is carried out

25 by electrophoresing the primer extension reaction products on ABI 373 Sequencer, followed by estimation of fluorescence intensities of the extended primer bands derived with the wild-type and mutant targets using the GeneScan™ 672 software.

30 The results of the primer extension analysis are shown below in Table II. Comparison of these results with those obtained by direct sequencing shows close similarities of the mutant frequencies obtained between the two methods. The primer extension method offers

35 greater sensitivity for detecting low frequency mutations, as illustrated in the following.

As mentioned earlier, mitochondrial mutations are maternally transmitted and segregate randomly in the next generation. A rare mutation in a maternal carrier who is asymptomatic can be inherited at a much higher frequency of occurrence by a process of repetitive segregation involving mitosis and meiosis. This appears to be the case for the NH0352 (mother) and NH0353 (son) pair. For this pair, heteroplasmy analysis for NH0353, a LHON patient, by the sequencing and the primer extension methods are in close agreement. In contrast, while the sequencing method is inadequate for detecting the presence of the mutation in the asymptomatic individual, NH0352, the primer extension method determines the presence of the mutation at a very low frequency. This provides a striking example of mitochondrial DNA inheritance, and the role of heteroplasmy in mitochondrial disease. Thus, the primer extension method of the present invention provides a rapid, convenient and non-isotopic approach for carrying out quantitative and multiplexed mutational analysis. The invention provides levels of sensitivity not achieved by current analytical methods and is immensely useful for analysis of complex genetic disorders.

TABLE II

	COMPARISON OF DNA SEQUENCING WITH PRIMER EXTENSION ASSAYS FOR THE DETECTION OF LHON 3460 MUTATION		
		% MUTANT DNA	
	PATIENT #	DNA SEQUENCING	PRIMER EXTENSION
	NH0004	100	100
	NH0006	0	0.1
5			
10	NH0013	39	33
	NH0087	78	79
	NH0088	75	70
	NH0089	94	89
	NH0098	0	0
15	NH0107	29	30
	NH0108	21	26
	NH0110	9	4
	NH0112	7	5
	NH0115	0	0.5
20	NH0129	0	0
	NH0182	6	10
	NH0183 ³	0	8
	NH0184	21	15
	NH0186	0	0
25	NH0187	25	35
	NH0352 ⁴	0	1.2
	NH0353 ²	55	52

Although the invention has been described with
 30 reference to the disclosed embodiments, those skilled in
 the art will readily appreciate that the specific
 examples provided herein are only illustrative of the

³ Heteroplasmic Family

⁴ NH0352 is the mother of NH0353

invention and not limitative thereof. It should be understood that various modifications can be made without departing from the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Fahy, Eoin D.
Ghosh, Soumitra
- (ii) TITLE OF INVENTION: Multiplexed Primer
Extension Methods
- (iii) NUMBER OF SEQUENCES: 25
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Kenyon & Kenyon
 - (B) STREET: 1025 Connecticut Avenue, N.W.
 - (C) CITY: Washington
 - (D) STATE: DC
 - (E) COUNTRY: USA
 - (F) ZIP: 20036
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3½ Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS 6.2
 - (D) SOFTWARE: WordPerfect 5.1
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/410,658
 - (B) FILING DATE: 24-MAR-1995
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Toffenetti, Judith L.
 - (B) REGISTRATION NUMBER: 39,048
 - (C) REFERENCE/DOCKET NUMBER: 2105/6
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (202) 429-1776
 - (B) TELEFAX: (202) 429-0796

49

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAGCCAACC CCATGGCCTC C

21

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTATTTAGT TGGGGCATT CAC

23

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACGTCCGGG AATTGTCATC TGTTTT

26

50

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AACTATCCTG CCCGCCA

17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGGCGTGAT CATGAAAGGT GATA

24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCATCCTTT ACATAACAGA CGAG

24

51

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCCCCTAA GATAGAGGAG A

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE C CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAGGGGGTT TTATATTGAT AATTG

25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGAAGAGGGG CGTTTGGTAT

20

52

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACTGGGAGA GATAGGAGAA GTAGG

25

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGGACTGGGA GAGATAGGAG AAGTA

25

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCTACGCCA AAATCCATTT C

21

53

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCCCCTATCA TAGAAGAGCT TATCA

25

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACTAGGATG ATGGCGGGCA

20

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGAGGGCGA TGAGGA

16

54

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCCAATTGA TTTGATGGTA

20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCCAATTGA TTTGATGGTA A

21

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CACCAATGGT ACTGAACCTA CGAG

24

55

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATTATTATAC GAATGGGGGC TTCA

24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTGATCCGTC CTAATCACAG CA

22

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCATCATAGG AGGCTTCATT CACTG

25

56

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: 1 linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TGATAGGATG TTTCATGTGG TGTATGC

27

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGTCAGAGG ATCAATCCCT C

21

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAGGGGGGAT CATAGAAG

18

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCTCTTTGGT GAAGAGTTTT ATGG

We Claim:

- 1 1. A method for simultaneous analysis of a genetic
2 mutation sequence and a corresponding wild-type sequence
3 within a biological sample comprising:
 - 4 (a) hybridizing a primer to a nucleic acid
5 suspected of containing a genetic mutation, wherein said
6 primer is hybridized 3' to said suspected mutation;
 - 7 (b) extending the primer in the presence of a
8 mixture of one to three deoxynucleoside triphosphates
9 and one to three chain terminating dideoxynucleoside
10 triphosphates selected such that the wild-type extension
11 product, the mutant-DNA derived extension product, and
12 the primer each are of different lengths.
 - 13 (c) separating said primer, said mutant DNA-
14 derived extension product and said wild-type extension
15 product on a molecular weight basis; and
 - 16 (d) identifying said mutant DNA-derived extension
17 product and said wild-type extension product.
- 1 2. The method of claim 1, wherein steps a) and b) are
2 repeated one or more times.
- 1 3. The method of claim 1, wherein said nucleic acid
2 suspected of containing the mutation is obtained by PCR
3 or RT-PCR.
- 1 4. The method of claim 1, wherein said primer further
2 comprises a detection moiety.
- 1 5. The method of claim 4, wherein said detection
2 moiety is selected from the group of a fluorescent label
3 and a radioisotope.
- 1 6. The method of claim 1, wherein said primer is
2 extended using a reverse transcriptase.

1 7. The method of claim 1, wherein said primer is
2 extended with a DNA polymerase.

1 8. The method of claim 7, wherein said DNA polymerase
2 selected from the group of a high fidelity polymerase
3 having a 3'-5' exonuclease activity and a thermostable
1 DNA polymerase.

1 9. The method of claim 1, wherein the relative amounts
2 of said mutant DNA-derived extension product and said
3 wild-type extension product are determined to quantify
4 the level of heteroplasmy of said genetic mutation.

1 10. A method for analyzing heteroplasmy of multiple
2 genetic mutations relative to corresponding wild-type
3 sequences within a sample comprising:

4 (a) hybridizing two or more differentiable primers
5 to a nucleic acid suspected of containing said genetic
6 mutations, wherein said primers are hybridized 3' to
7 said suspected mutations;

8 (b) extending said primers in the presence of a
9 mixture of one to three deoxynucleoside triphosphates
10 and one to three chain terminating dideoxynucleoside
11 triphosphates such that for each of the two or more
12 differentiable primers, the corresponding wild-type
13 extension product and the corresponding mutant-DNA
14 derived extension product differ in length from each
15 other and the primer;

16 (c) separating said primers, said mutant DNA-
17 derived extension products and said wild-type extension
18 products on a molecular weight basis; and

19 (d) identifying said mutant DNA-derived extension
20 products and said wild-type extension products.

1 11. The method of claim 10, wherein step b) is repeated
2 one or more times.

- 1 12. The method of claim 10, wherein said two or more
2 primers are hybridized to said DNA fragment
3 simultaneously.
- 1 13. The method of claim 10, wherein said two or more
2 primers are hybridized and extended separately and are
3 combined before conducting said separation step.
- 1 14. The method of claim 10, wherein said DNA fragment
2 suspected of containing the mutation is obtained by PCR
3 or RT-PCR.
- 1 15. The method of claim 10, wherein said two or more
2 primers further comprise a detection moiety.
- 1 16. The method of claim 15, wherein said detection
2 moiety is selected from the group of a fluorescent label
3 and a radioisotope.
- 1 17. The method of claim 15, wherein said two or more
2 differentiable primers are selected from the group of
3 primers having different lengths and primers comprising
4 fluorescent labels with different excitation or emission
5 maxima.
- 1 18. The method of claim 10, wherein said two or more
2 primers are extended with a DNA polymerase.
- 1 19. The method of claim 18, wherein said DNA polymerase
2 is selected from the group of a high fidelity polymerase
3 having 3'-5' exonuclease activity and a thermostable DNA
4 polymerase.
- 1 20. A method for simultaneously determining the
2 presence of related polynucleotide sequences in a
3 nucleic acid sample comprising:

- 4 (a) providing a nucleic acid sample suspected of
5 containing related polynucleotides with identical
6 regions and divergent regions;
7 (b) providing a primer that is complementary to
8 said identical regions;
9 (c) hybridizing said primer to said identical
10 regions;
11 (d) extending said primer in the presence of a
12 polymerase and a nucleotide mixture containing from one
13 to three dNTPs, such that an extension product of unique
14 length is formed for each related polynucleotide; and
15 (e) separating said primer and said extension
16 products based on their respective lengths.

1 21. The method of claim 20, wherein said divergent
2 regions differ by a single substituted nucleotide, by
3 the addition of one or more nucleotides, or by the
4 deletion of one or more nucleotides.

1 22. The method of claim 20, wherein said nucleotide
2 mixture contains two or three dNTPs and contains no
3 chain terminating, base-pairing entities.

1 23. The method of claim 22, wherein one of said dNTPs
2 is labeled with a detection moiety.

1 24. The method of claim 20, wherein said nucleotide
2 mixture contains one to three dNTPs and one to three
3 chain terminating, base-pairing entities.

1 25. The method of claim 24, wherein one of said dNTPs
2 and said chain terminating, base-pairing entities is
3 labeled with a detection moiety.

1 26. The method of claim 20, wherein said primer and
2 said extension products are separated by a technique

3 selected from the group of gel electrophoresis and mass
4 spectroscopy.

1 27. The method of claim 20, wherein steps c) and d) are
2 repeated one or more times.

1 28. The method of claim 20, wherein said nucleic acid
2 suspected of containing the mutation is obtained by PCR
3 or RT-PCR.

1 29. The method of claim 20, wherein said primer is
2 labeled with a detection moiety.

1 30. The method of claim 20, wherein said detection
2 moiety is selected from the group of a fluorescent label
3 and a radioisotope.

1 31. The method of claim 20, wherein said primer is
2 extended with a reverse transcriptase.

1 32. The method of claim 20, wherein said primer is
2 extended with a DNA polymerase.

1 33. The method of claim 32, wherein said DNA polymerase
2 is selected from a high fidelity polymerase having 3'-5'
3 exonuclease activity and a thermostable DNA polymerase.

1 34. A kit for simultaneously determining the presence
2 of at least two related polynucleotide sequences in a
3 nucleic acid sample, said related polynucleotides having
4 identical regions and divergent regions, said kit
5 comprising:
6 a primer that is complementary to said identical
7 regions;
8 a polymerase; and

9 a nucleotide mixture containing one to three dNTPs
10 such that an extension product of unique length is
11 formed for each related polynucleotide.

1 35. The kit of claim 34, wherein said nucleotide
2 mixture contains two or three dNTPs and contains no
3 chain terminating, base-pairing entities.

1 36. The kit of claim 34, wherein said nucleotide
2 mixture contains one to three dNTPs and one to three
3 chain terminating, base-pairing entities.

1 37. A kit for simultaneously analyzing a genetic
2 mutation and a corresponding wild-type sequence within a
3 sample comprising:

4 a primer; a polymerase; and a mixture of one to
5 three deoxynucleoside triphosphates and one to three
6 chain terminating dideoxynucleoside triphosphates
7 selected such that the wild-type extension product and
8 the mutant-DNA derived extension product, and the primer
9 are of different lengths.

1 38. The method of claim 1, wherein said mutation is
2 found in a gene selected from the COX1 gene and the COX2
3 gene.

1 39. The method of claim 1, wherein said mutation is
2 found at a codon selected from codon 155 of COX1, codon
3 167 of COX1, codon 178 of COX1, codon 193 of COX1, codon
4 194 of COX1, codon 415 of COX1.

1 40. The method of claim 1, wherein said mutation is
2 found at a codon selected from codon 20 of COX2, codon
3 22 of COX2, codon 68 of COX2, codon 71 of COX2, codon 74
4 of COX2, codon 90 of COX2, codon 95 of COX2, codon 110
5 of COX2 and codon 146 of COX2.

1 41. The method of claim 1, wherein said mutation is
2 indicative of a disease state selected from Alzheimer's
3 disease and Leber Hereditary Optic Neuropathy.

1 42. The kit of claim 37, selected from the group of:
2 (a) a kit for the analysis of a genetic mutation
3 of codon 155 of COX1 wherein said primer is 5'-
4 TGGCCCCTAAGATAGAGGAGA-3' (SEQ. ID NO. 7); and said
5 mixture contains a deoxynucleoside triphosphate portion
6 consisting essentially of dCTP and a dideoxynucleoside
7 triphosphate portion consisting essentially of ddATP and
8 ddTTP;

9 (b) a kit for the analysis of a genetic mutation
10 of codon 167 of COX1 wherein said primer is 5'-
11 GCAGGGGGTTTTATATTGATAATTG-3' (SEQ. ID NO. 8); and said
12 mixture contains a deoxynucleoside triphosphate portion
13 consisting essentially of dTTP and a dideoxynucleoside
14 triphosphate portion consisting essentially of ddCTP and
15 ddGTP;

16 (c) a kit for the analysis of a genetic mutation
17 of codon 178 of COX1 wherein said primer is 5'-
18 CGAAGAGGGGCGTTTGGTAT-3' (SEQ. ID NO. 9); and said
19 mixture contains a deoxynucleoside triphosphate portion
20 consisting essentially of dTTP and a dideoxynucleoside
21 triphosphate portion consisting essentially of ddATP and
22 ddGTP;

23 (d) a kit for the analysis of a genetic mutation
24 of codon 193 of COX1 wherein said primer is 5'-
25 GACTGGGAGAGATAGGAGAAGTAGG-3' (SEQ. ID NO. 10); and said
26 mixture contains a deoxynucleoside triphosphate portion
27 consisting essentially of dATP and dCTP and a
28 dideoxynucleoside triphosphate portion consisting
29 essentially of ddGTP and ddTTP;

30 (e) a kit for the analysis of a genetic mutation
31 of codon 194 of COX1 wherein said primer is 5'-
32 AGGACTGGGAGAGATAGGAGAAGTA-3' (SEQ. ID NO. 11); and said
33 mixture contains a deoxynucleoside triphosphate portion

34 consisting essentially of dGTP and a dideoxynucleoside
35 triphosphate portion consisting essentially of ddATP;
36 and
37 (f) a kit for the analysis of a genetic mutation
38 of codon 415 of COX1 wherein said primer is 5'-
39 ACCTACGCCAAAATCCATTTC-3' (SEQ. ID NO. 12); and said
40 mixture contains a deoxynucleoside triphosphate portion
41 consisting essentially of dATP and a dideoxynucleoside
42 triphosphate portion consisting essentially of ddCTP and
43 ddGTP.

1 43. The kit of claim 37 selected from the group of:
2 (a) a kit for the analysis of a genetic mutation
3 of codon 20 of COX2 wherein said primer is 5'-
4 AGGGCGTGATCATGAAAGGTGATA-3' (SEQ. ID NO. 5); and said
5 mixture contains a deoxynucleoside triphosphate portion
6 consisting essentially of dATP and a dideoxynucleoside
7 triphosphate portion consisting essentially of ddGTP;
8 (b) a kit for the analysis of a genetic mutation
9 of codon 22 of COX2 wherein said primer is 5'-
10 TCCCCTATCATAGAAGAGCTTATCA-3' (SEQ. ID NO. 13); and said
11 mixture contains a deoxynucleoside triphosphate portion
12 consisting essentially of dCTP and a dideoxynucleoside
13 triphosphate portion consisting essentially of ddTTP;
14 (c) a kit for the analysis of a genetic mutation
15 of codon 68 of COX2 wherein said primer is 5'-
16 GACTAGGATGATGGCGGGCA-3' (SEQ. ID NO. 14); and said
17 mixture contains a deoxynucleoside triphosphate portion
18 consisting essentially of dGTP and a dideoxynucleoside
19 triphosphate portion consisting essentially of ddATP;
20 (d) a kit for the analysis of a genetic mutation
21 of codon 71 of COX2 wherein said primer is 5'-
22 AACTATCCTGCCCCCA-3' (SEQ. ID NO. 4); and said mixture
23 contains a deoxynucleoside triphosphate portion
24 consisting essentially of dTTP and a dideoxynucleoside
25 triphosphate portion consisting essentially of ddCTP;

- 26 (e) a kit for the analysis of a genetic mutation
27 of codon 74 of COX2 wherein said primer is 5'-
28 GGGAGGGGCGATGAGGA-3' (SEQ. ID NO. 15); and said mixture
29 contains a deoxynucleoside triphosphate portion
30 consisting essentially of dCTP and a dideoxynucleoside
31 triphosphate portion consisting essentially of ddTTP;
- 32 (f) a kit for the analysis of a genetic mutation
33 of codon 90 of COX2 wherein said primer is 5'-
34 CGCATCCTTTACATAACAGACGAG-3' (SEQ. ID NO. 6); and said
35 mixture contains a deoxynucleoside triphosphate portion
36 consisting essentially of dGTP and a dideoxynucleoside
37 triphosphate portion consisting essentially of ddATP and
38 ddTTP;
- 39 (g) a kit for the analysis of a genetic mutation
40 of codon 95 of COX2 wherein said primer is 5'-
41 GGCCAATTGATTTGATGGTA-3' (SEQ. ID NO. 16); and said
42 mixture contains a deoxynucleoside triphosphate portion
43 consisting essentially of dATP and a dideoxynucleoside
44 triphosphate portion consisting essentially of ddGTP;
- 45 (h) a kit for the analysis of a genetic mutation
46 of codon 95 of COX2 wherein said primer is 5'-
47 GGCCAATTGATTTGATGGTAA-3' (SEQ. ID NO. 17); and said
48 mixture contains a deoxynucleoside triphosphate portion
49 consisting essentially of dGTP and a dideoxynucleoside
50 triphosphate portion consisting essentially of ddATP and
51 ddTTP;
- 52 (i) a kit for the analysis of a genetic mutation
53 of codon 95 of COX2 wherein said primer is 5'-
54 GGCCAATTGATTTGATGGTAA-3' (SEQ. ID NO. 17); and said
55 mixture contains a deoxynucleoside triphosphate portion
56 consisting essentially of dGTP and a dideoxynucleoside
57 triphosphate portion consisting essentially of ddATP;
- 58 (j) a kit for the analysis of a genetic mutation
59 of codon 110 of COX2 wherein said primer is 5'-
60 CACCAATGGTACTGAACCTACGAG-3' (SEQ. ID NO. 18); and said
61 mixture contains a deoxynucleoside triphosphate portion
62 consisting essentially of dATP and dTTP and a

63 dideoxynucleoside triphosphate portion consisting
64 essentially of ddCTP and ddGTP; and
65 (k) a kit for the analysis of a genetic mutation
66 of codon 146 of COX2 wherein said primer is 5'-
67 ATTATTATACGAATGGGGGCTTCA-3' (SEQ. ID NO. 19); and said
68 mixture contains a deoxynucleoside triphosphate portion
69 consisting essentially of dATP and dTTP and a
70 dideoxynucleoside triphosphate portion consisting
71 essentially of ddCTP and ddGTP.

1 44. The kit of claim 37, selected from the group of:

2 (a) a kit for the analysis of a genetic mutation
3 of codon 155 of COX1 wherein said primer is 5'-
4 TGGCCCCTAAGATAGAGGAGA-3' (SEQ. ID NO. 7); and said
5 mixture contains a deoxynucleoside triphosphate portion
6 consisting essentially of dTTP and a dideoxynucleoside
7 triphosphate portion consisting essentially of ddATP and
8 ddCTP;

9 (b) a kit for the analysis of a genetic mutation
10 of codon 167 of COX1 wherein said primer is 5'-
11 GCAGGGGGTTTTATATTGATAATTG-3' (SEQ. ID NO. 8); and said
12 mixture contains a deoxynucleoside triphosphate portion
13 consisting essentially of dCTP and a dideoxynucleoside
14 triphosphate portion consisting essentially of ddTTP;

15 (c) a kit for the analysis of a genetic mutation
16 of codon 178 of COX1 wherein said primer is 5'-
17 CGAAGAGGGGCGTTTGGTAT-3' (SEQ. ID NO. 9); and said
18 mixture contains a deoxynucleoside triphosphate portion
19 consisting essentially of dATP and a dideoxynucleoside
20 triphosphate portion consisting essentially of ddGTP and
21 ddTTP;

22 (d) a kit for the analysis of a genetic mutation
23 of codon 193 of COX1 wherein said primer is 5'-
24 CTGATCCGTCCTAATCACAGCA-3' (SEQ. ID NO. 20); and said
25 mixture contains a deoxynucleoside triphosphate portion
26 consisting essentially of dGTP and dTTP and a

27 dideoxynucleoside triphosphate portion consisting
28 essentially of ddATP and ddCTP;

29 (e) a kit for the analysis of a genetic mutation
30 of codon 194 of COX1 wherein said primer is 5'-
31 AGGACTGGGAGAGATAGGAGAAGTA-3' (SEQ. ID NO. 11); and said
32 mixture contains a deoxynucleoside triphosphate portion
33 consisting essentially of dATP and a dideoxynucleoside
34 triphosphate portion consisting essentially of ddGTP;
35 and

36 (f) a kit for the analysis of a genetic mutation
37 of codon 415 of COX1 wherein said primer is 5'-
38 ACCTACGCCAAAATCCATTTTC-3' (SEQ. ID NO. 12); and said
39 mixture contains a deoxynucleoside triphosphate portion
40 consisting essentially of dGTP and a dideoxynucleoside
41 triphosphate portion consisting essentially of ddATP and
42 ddCTP.

1 45. The kit of claim 37, selected from the group of:

2 (a) a kit for the analysis of a genetic mutation
3 of codon 20 of COX2 wherein said primer is 5'-
4 AGGGCGTGATCATGAAAGGTGATA-3' (SEQ. ID NO. 5); and said
5 mixture contains a deoxynucleoside triphosphate portion
6 consisting essentially of dGTP and a dideoxynucleoside
7 triphosphate portion consisting essentially of ddATP and
8 ddCTP;

9 (b) a kit for the analysis of a genetic mutation
10 of codon 22 of COX2 wherein said primer is 5'-
11 TCCCCTATCATAGAAGAGCTTATCA-3' (SEQ. ID NO. 13); and said
12 mixture contains a deoxynucleoside triphosphate portion
13 consisting essentially of dTTP and a dideoxynucleoside
14 triphosphate portion consisting essentially of ddCTP;

15 (c) a kit for the analysis of a genetic mutation
16 of codon 68 of COX2 wherein said primer is 5'-
17 GACTAGGATGATGGCGGGCA-3' (SEQ. ID NO. 14); and said
18 mixture contains a deoxynucleoside triphosphate portion
19 consisting essentially of dATP and a dideoxynucleoside
20 triphosphate portion consisting essentially of ddGTP;

21 (d) a kit for the analysis of a genetic mutation
22 of codon 71 of COX2 wherein said primer is 5'-
23 AACTATCCTGCCCCGCCA-3' (SEQ. ID NO. 4); and said mixture
24 contains a deoxynucleoside triphosphate portion
25 consisting essentially of dCTP and a dideoxynucleoside
26 triphosphate portion consisting essentially of ddATP and
27 ddTTP;

28 (e) a kit for the analysis of a genetic mutation
29 of codon 74 of COX2 wherein said primer is 5'-
30 GGGAGGGCGATGAGGA-3' (SEQ. ID NO. 15); and said mixture
31 contains a deoxynucleoside triphosphate portion
32 consisting essentially of dTTP and a dideoxynucleoside
33 triphosphate portion consisting essentially of ddATP and
34 ddCTP;

35 (f) a kit for the analysis of a genetic mutation
36 of codon 90 of COX2 wherein said primer is 5'-
37 CGCATCCTTTACATAACAGACGAG-3' (SEQ. ID NO. 6); and said
38 mixture contains a deoxynucleoside triphosphate portion
39 consisting essentially of dATP and a dideoxynucleoside
40 triphosphate portion consisting essentially of ddGTP and
41 ddTTP;

42 (g) a kit for the analysis of a genetic mutation
43 of codon 95 of COX2 wherein said primer is 5'-
44 GGCCAATTGATTTGATGGTA-3' (SEQ. ID NO. 16); and said
45 mixture contains a deoxynucleoside triphosphate portion
46 consisting essentially of dGTP and a dideoxynucleoside
47 triphosphate portion consisting essentially of ddATP;

48 (h) a kit for the analysis of a genetic mutation
49 of codon 95 of COX2 wherein said primer is 5'-
50 GGCCAATTGATTTGATGGTAA-3' (SEQ. ID NO. 17); and said
51 mixture contains a deoxynucleoside triphosphate portion
52 consisting essentially of dTTP and a dideoxynucleoside
53 triphosphate portion consisting essentially of ddGTP;

54 (i) a kit for the analysis of a genetic mutation
55 of codon 110 of COX2 wherein said primer is 5'-
56 CACCAATGGTACTGAACCTACGAG-3' (SEQ. ID NO. 18); and said
57 mixture contains a deoxynucleoside triphosphate portion

58 consisting essentially of dGTP and dTTP and a
59 dideoxynucleoside triphosphate portion consisting
60 essentially of ddATP and ddCTP; and
61 (j) a kit for the analysis of a genetic mutation
62 of codon 146 of COX2 wherein said primer is 5'-
63 ATTATTATACGAATGGGGGCTTCA-3' (SEQ. ID NO. 19); and said
64 mixture contains a deoxynucleoside triphosphate portion
65 consisting essentially of dATP and dCTP and a
66 dideoxynucleoside triphosphate portion consisting
67 essentially of ddGTP and ddTTP.

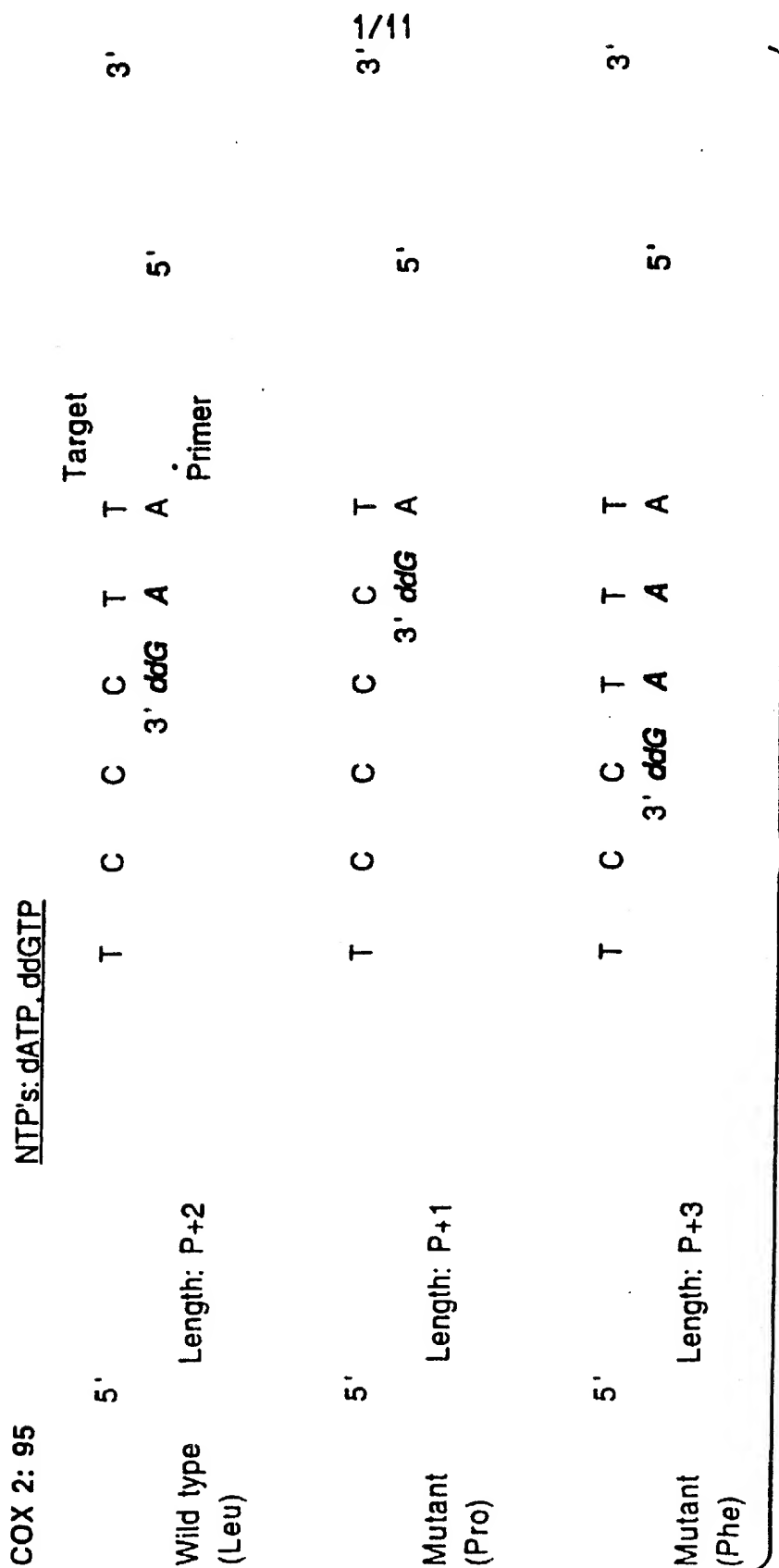


FIG. 1

2 / 11

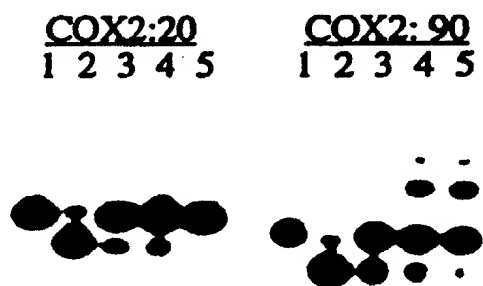


FIG.2

3/11

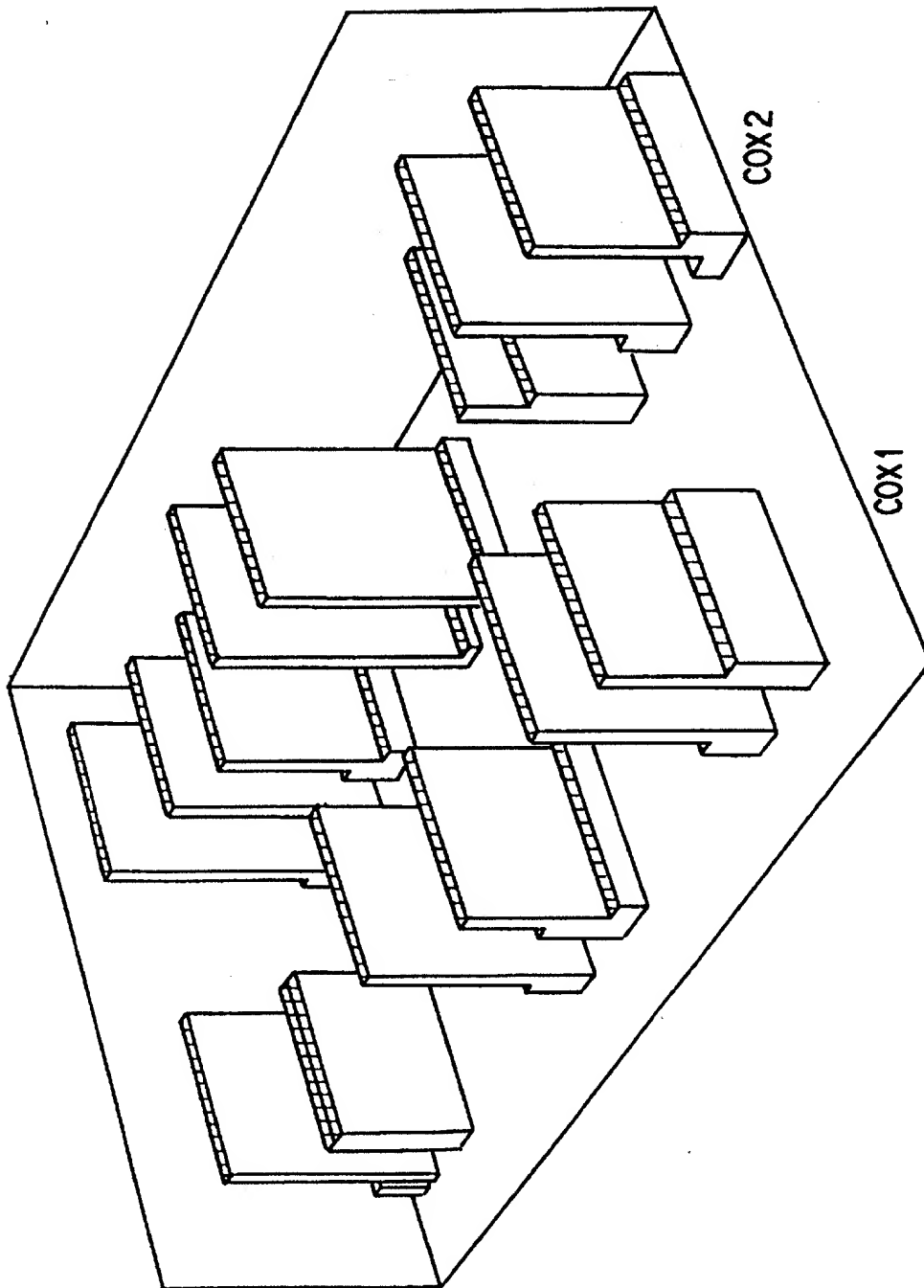


FIG. 3

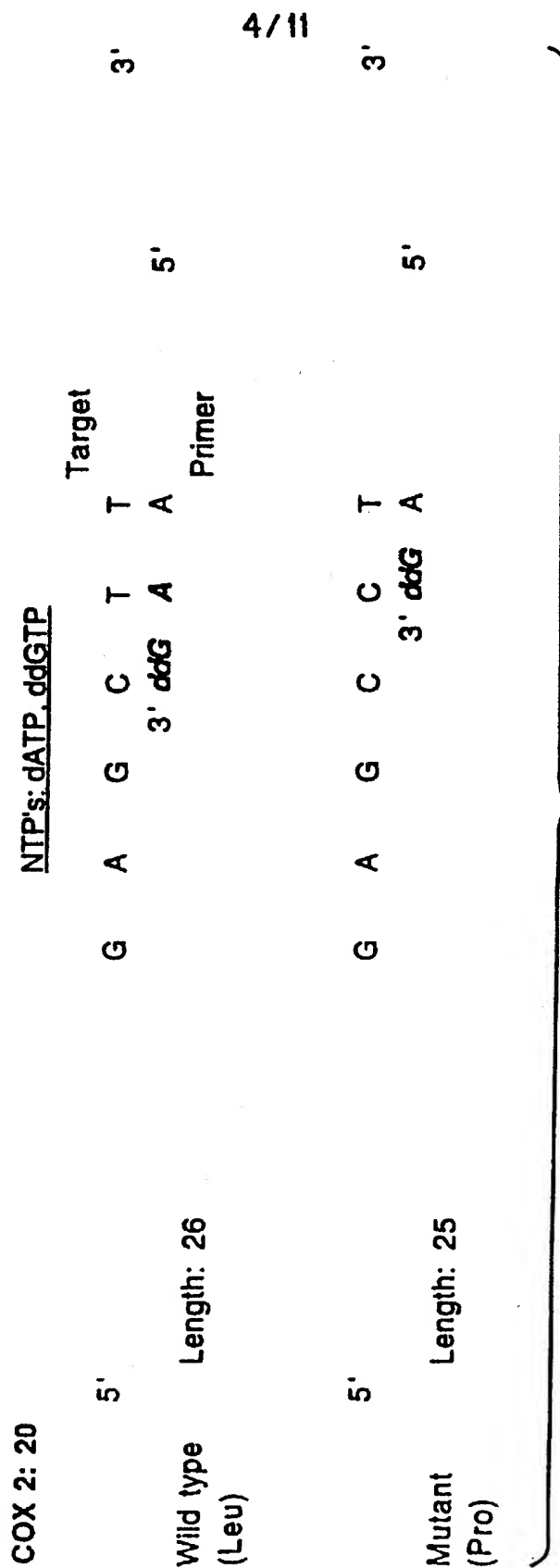
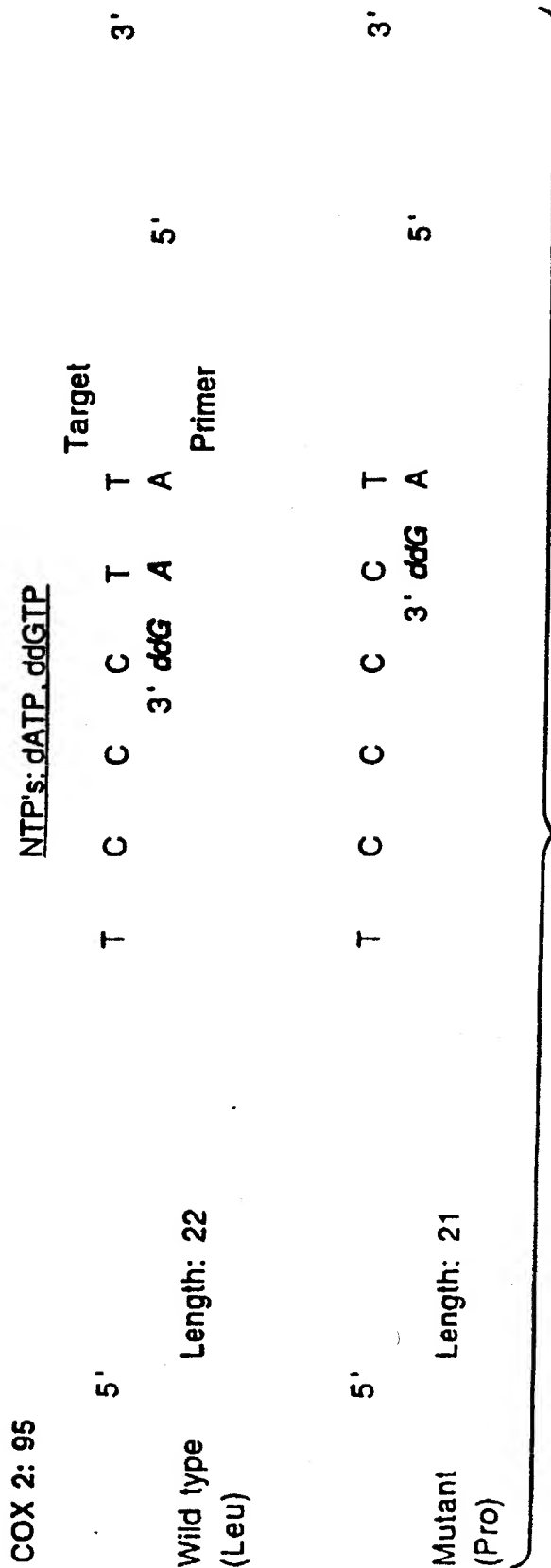


FIG.4A

5/11



6/11

COX2 #20

COX2 #95



FIG.5A

7/11

MULTIPLEX

COX 2 #20 AND #95



FIG.5B

8/11

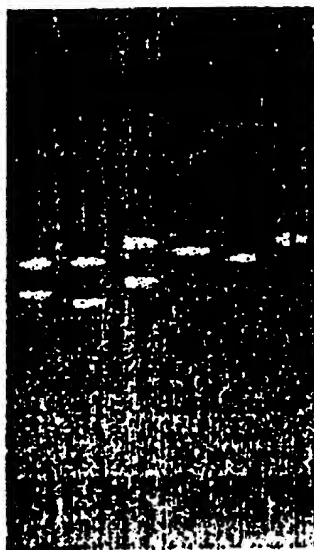


FIG.5C

9/11

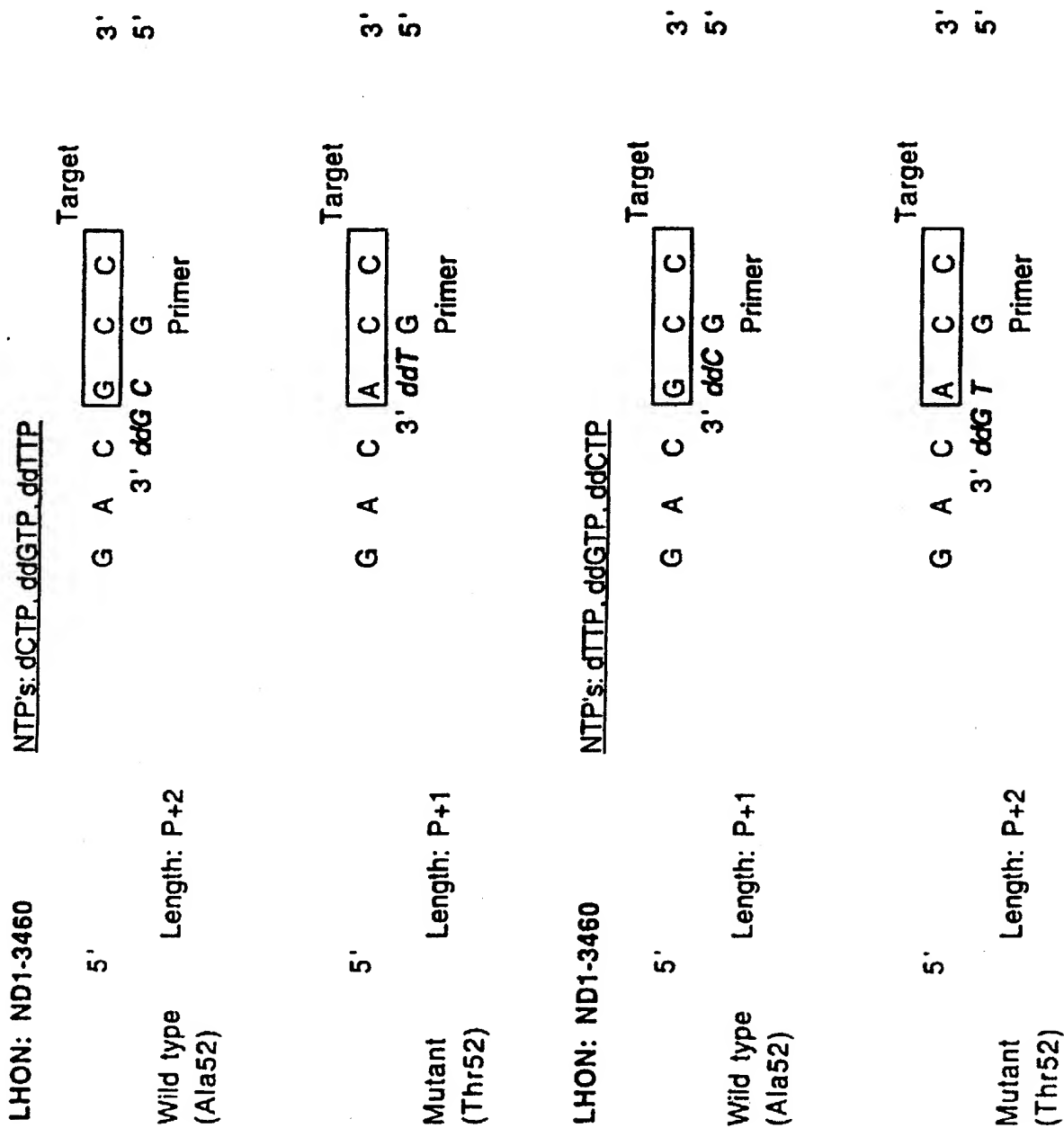


FIG.6

10/11

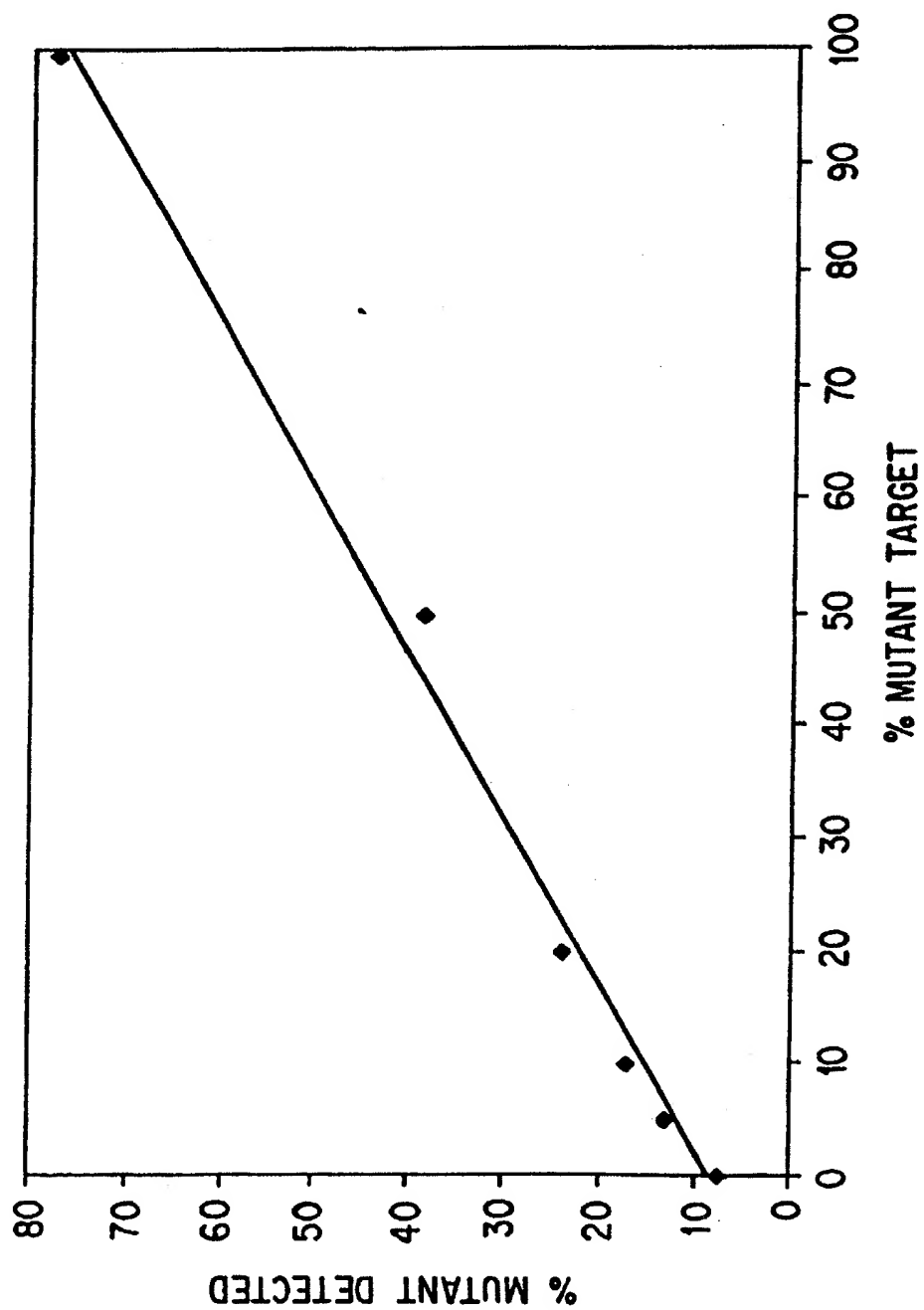


FIG. 7

11/11

Loading 4

Loading 3

Loading 2

Loading 1

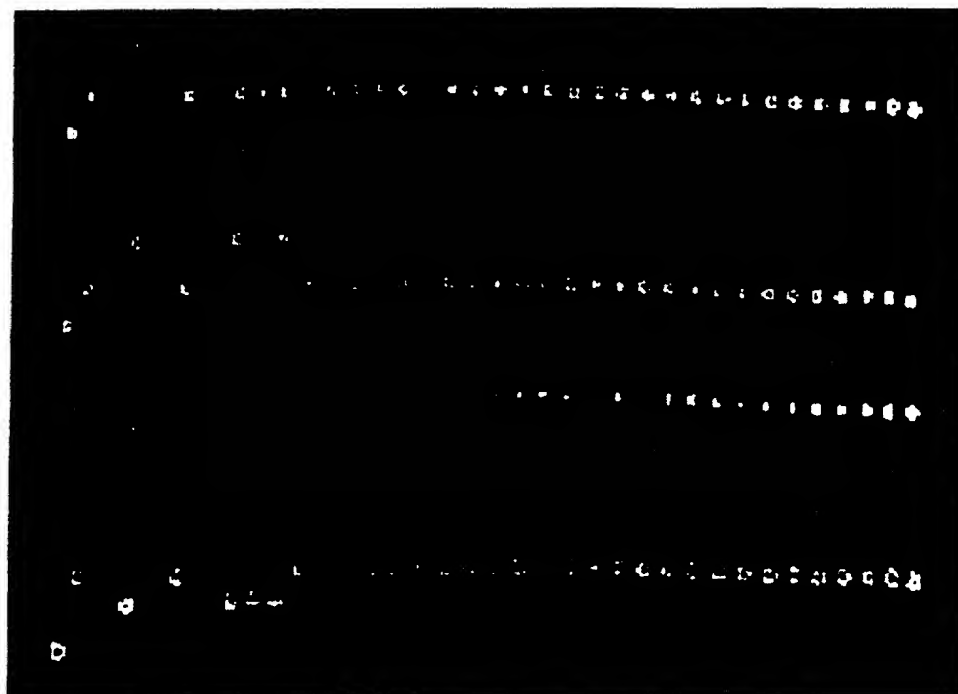


FIG.8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02045

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68, 1/70; C07H 21/02, 21/04
US CL : 435/5 6, 91.2; 536/24.3, 24.31, 24.32, 24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 91.2; 536/24.3, 24.31, 24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	PALEJWALA et al. Quantitative multiplex sequence analysis of mutational hot spots: Frequency and specificity of mutations induced by a site-specific ethenocytosine in m13 viral DNA. Biochemistry. 1993, Vol. 32, pages 4105-4111.	1,2,4,5, 7-9,20,21, 24-27,29, 30,32,33 ----- 3,6,10-19, 22,23,28, 31,41
Y	PARK. 'PCR in the diagnosis of retinoblastoma.' In: PCR protocols: A guide to methods and applications, Edited by M. Innis et al. New York:Academic Press, Inc., 1990, pages 407-415.	3, 6, 8, 14, 18, 19, 28, 3 1,32,33
X	New England Biolabs Catalog. Circum Vent Thermal Cycle DNA Sequencing Kit. 1993/34, page 110.	34-37

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
*A	document defining the general state of the art which is not considered to be of particular relevance		
*E	earlier document published on or after the international filing date	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O	document referring to an oral disclosure, use, exhibition or other means		
*P	document published prior to the international filing date but later than the priority date claimed	*Z	document member of the same patent family

Date of the actual completion of the international search 11 JUNE 1996	Date of mailing of the international search report 24 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY FREDMAN Telephone No. (703) 308-6196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02045

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	WO 90/09455 A1 (GENECO PTY LTD) 23 August 1990, pages 1-57, especially pages 31-33.	34-37 ----- 1-33, 41
Y	BLASZYK et al. Rapid and efficient screening for p53 gene mutations by dideoxy fingerprinting. Biotechniques. February 1995, Vol. 18, No. 2, pages 256, 258-260, see entire document.	1-33, 41
Y	SARKAR et al. Dideoxy fingerprinting (ddF): A rapid and efficient screen for the presence of mutations. Genomics. 1992, Vol. 13, pages 441-443, see entire document.	1-33, 41
A	EP 0 412 883 A1 (D. COHEN et al) 02 August 1990, see entire document.	1-33, 41
Y	HUOPONEN et al. The spectrum of mitochondrial DNA mutations in families with Leber hereditary optic neuroretinopathy. Human Genetics. 1993, Volume 92, pages 379-384.	41

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/02045

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 38-40 and 42-45
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02045

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPLUS, WPIDS

search terms: mutation, mutant, polymerase, size, length, mobility, electrophoresis, deoxynucleotide, dideoxynucleotide, dntp, ddntp, extension, primer

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

Claims 38-40 and 42-45 were not searched because PCT Rule 13ter requires that sequence listings be submitted for search with international cases. Since claims 38-40 and 42-45 depend for novelty on specific sequences which were not submitted in machine readable form to the International Searching Authority, these claims cannot be meaningfully searched.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-33 and 41, drawn to methods for mutation analysis.

Group II, claim(s) 34-37, drawn to kits containing nucleic acids and polymerases.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The invention of Group I is drawn to a method of analysis of genetic mutations, which method is designed to work with any specific gene mutations and is not limited by the claims or the description to the specific COX2 gene. The invention of Group II includes as it's narrowest embodiment primers drawn to the COX2 gene in a kit, which could be used in a variety of methods other than the method of claim 1 including PCR. There is no special technical feature which links the two groups since the broad method is not linked to the narrow specific primers.